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PIN1 as new oncogene in ovarian cancer: function and chemical inhibition

Settore scientifico-disciplinare: ingegneria chimica

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INDEX

Abstract

PIN1 is a member of peptidylprolyl isomerases family that binds phosphoproteins and catalyzes the rapid *cis–trans* isomerization of proline peptidyl bonds, resulting in an alteration of protein structure, function and stability. PIN1 was found overexpressed in human cancers and promotes tumorigenesis, althought, depending on the cellular context, it also acts as a tumor suppressor.

Here, we found that PIN1 is overexpressed in serous ovarian cancer patients and show that PIN1 is a new oncogene in this type of cancer. PIN1 inhibition in different ovarian cancer cell lines affected cancer cell viability and promoted cell death via activation of apoptotic program.

Since few therapies are effective against ovarian cancer because almost all patients develop resistance to conventional treatments, we hypothesized that PIN1 could be a potential therapeutic target.

We identified a small molecule (VS10) that selectively binds and inhibits PIN1 as demonstrated by decreased levels of the PIN1 downstream targets β-catenin, cyclin D1 and pSer473-Akt in VS10-treated cells. Moreover, VS10 reduced the viability of four ovarian cancer cell lines.

These results suggest that VS10 might be an efficient and selective PIN1 inhibitor, which could offer a new opportunity for treating PIN1-overexpressing tumors.

1. Introduction

1.1. Ovarian cancer

1.1.1. Classification

GLOBOCAN has estimated 295,414 new ovarian cancer (OC) cases worldwide in 2018 and that 184,799 patients will die for the disease (Bray et al., 2018).

In general, the incidence rate and mortality has declined in the last decades, however it is highest in the developed coutries including North America and Europe, intermediate in South America and lowest in Asia and Africa (American Cancer Society, 2018).

The disease is typically diagnosed at late stage and only the 15% of cases when it is a localized tumor (American Cancer Society, 2018).

To date two hypothesis have been emerged to explain OC initiation: the incessant ovulation hypothesis and the gonadotropin hypothesis. The incessant ovulation hypotesys suggests that a major number of ovulation cycles involves increased cell divisions, thus a major risk to accumulate mutations; the gonadotropin hypotesys suggests that the luteinizing hormone and the follicle-stimulating hormone, involved in ovulation cycles, increase the risk of OC. According to these hypothesis, the age at the menarca and menopause increase cancer risk, whereas pregnancy and lactation have a protective effect. Several gynaecological conditions (inflammations, endometriosis…) are associated with OC, whereas some surgical procedures (hysterectomy, tubal ligation…) decrease the risk. In the last decades, it has been established tha the use of oral contraceptives has a protective effect, whereas no clear evidence have emerged about the hormone replacement therapy correlation with OC risk. As for other diseases, obesity is a risk factor, whereas the exercise and physical activity have several benefits. The association between OC and diet, alchol consumption or smoking needs to be further investigated (Reid, Permuth, & Sellers, 2017).

Despite the recent progress in OC research, the etiology of this lethal disease is not completely understood. Both benign or malignant tumors origin from epithelial, stromal or germ line cells. (Sankaranarayanan & Ferlay, 2006).
The epithelial OC (EOC) are the most common (90%), followed by the sex cord-stromal tumors (7%) and germ cell tumors (3%) (Romero & Bast, 2012).

The EOC can be classified in five different histologic subtypes (hystotypes):

- the high-grade serous epithelial OC (HGS-EOC; 70%);
- the endometrioid OC (ENOC; 10%);
- the clear cell OC (CCOC; 10%);
- the mucinous OC(MOC; 3%);
- the low-grade serous epithelial OC (LGS-EOC; <5%)

They differ in their cellular origin, pathogenesis, molecular alterations, gene expression, and prognosis. (Prat, 2012).

The CCOC and ENOC are suggested to originate from the endometriotic cysts associated with endometriosis (Veras et al., 2009); the MOC originates from transitional cell nests at the tubal-mesothelial junction (Seidman & Khedmati, 2008), whereas the HGS-EOC and LGS-EOC seem to derive from the surface of the ovary and/or the distal fallopian tube epithelium (Piek et al., 2001) (Kuhn et al., 2012) (Li et al., 2011).

The LGS-EOC, CCOC, low-grade ENOC and MOC are designed as Type I OCs: they maintain borderline tumor characteristics, grow slowly, are diagnosed in an early stage (I or II) and are resistant to chemotherapy, although they respond to hormonal treatment.
HGS-EOCs and high-grade ENOC are classified as Type II OCs: they are the most aggressive OC subtypes, grow rapidly, respond to chemotherapy but less to hormonal treatment (Romero & Bast, 2012).

Figure 1 summarizes Type I and Type II OCs.

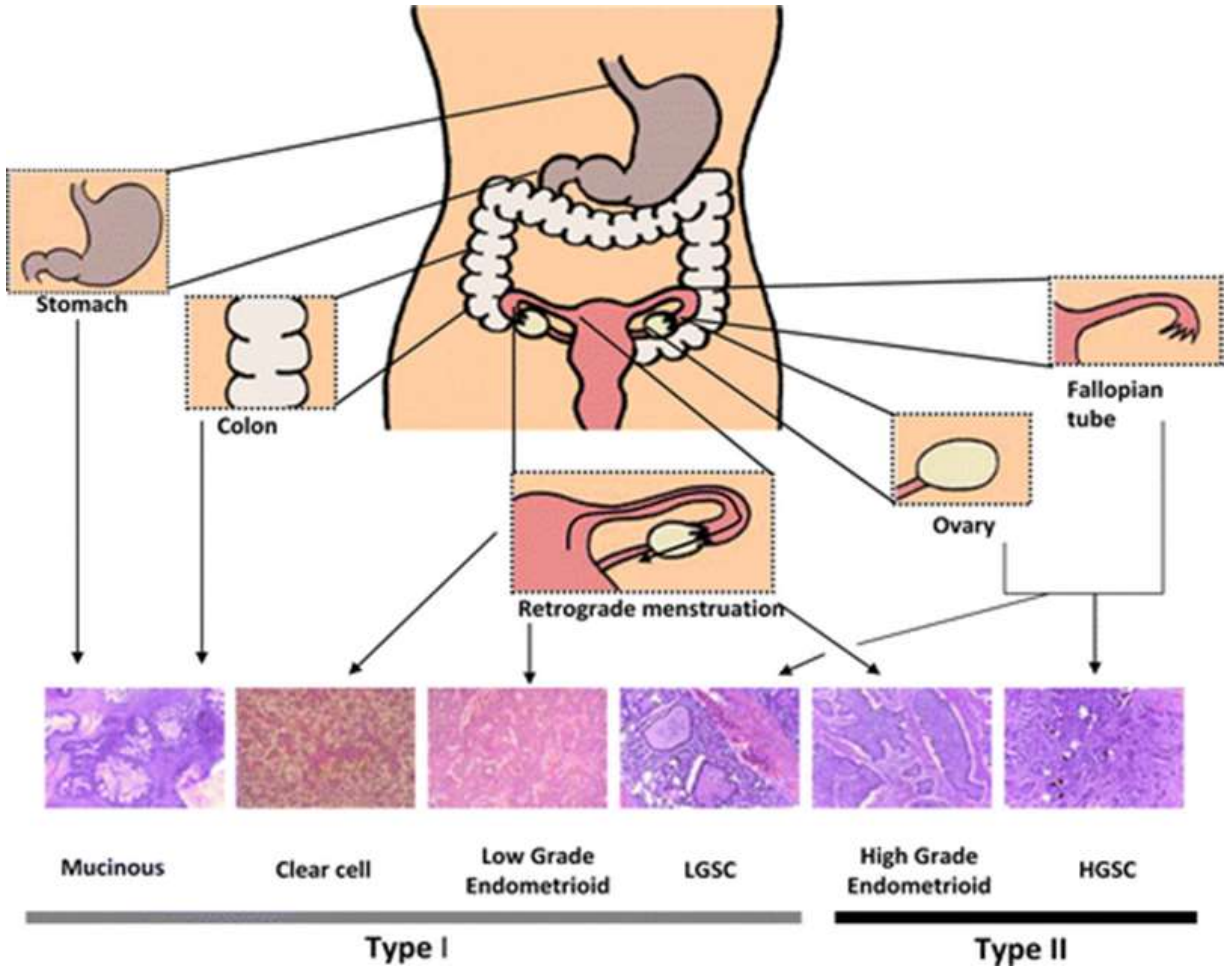


Figure 1. *Type I and Type II ovarian cancer subtypes and their sites of origin.* (Romero & Bast, 2012).

1.1.2. Mutated genes and cancer-driving pathways in ovarian cancer

The mutation spectrum of Type I and II OC subtypes are completely different each other.

In general, the Type I tumors present mutation in BRAF and KRAS oncogenes (LGS-EOC and MOC) or PTEN (ENOC) (Shih & Kurman, 2004).

The 96% of HGS-EOCs are characterized by TP53 mutations (TCGA, 2011). In addition, in HGS-EOC they were found some somatic mutations in 9 additional genes including NF1, BRCA1, BRCA2, RB1, and CDK12, significant focal DNA copy number aberrations and promoter methylation in 168 genes (TCGA, 2011). Among the focal DNA copy number aberrations, PIN1 (peptidylprolyl *cis/trans* isomerase, NIMA-interacting 1) protein was found overexpressed.

The proteins codified by the altered genes are involved in the main molecular pathways that result deregulated in cancer. Understanding the molecular mechanisms that govern HGS-EOC initiation and progression might provide new opportunities for HGS-EOC treatment.

Retinoblastoma protein (RB) pathway was found altered in 67% of HGS-EOC cases (TCGA, 2011). Rb exists as a phosphorylated protein (pRb), regulated through hypo/hyper-phosphorylation by cyclin-dependent kinase (CDK). When hypophosphorylated, Rb binding to the transcription factor E2F inhibits E2F transcriptional activity. As a result, the cell cycle is arrested; Rb hyperphosphorylation allows to E2F release and expression of genes which control S-phase entry (Johnson & Schneider-Broussard, 1998). In cancer Rb was aberrantly hyperphosphorilated and controls cancer cell proliferation (Chinnam & Goodrich, 2011).

Ras protein activation regulates the phophatidylinositol 3 Kinase (PI3K) activity. The PI3K is an enzyme composed of a p110 catalytic subunit and a p85 regulatory subunit. The p110 subunit of PI3K phosphorylates the phosphatidylinositol-4,5-bisphosphate (PIP2) to the active second messenger, PIP3, which recruits to the plasma membrane the serine/threonine Akt, also known as protein kinase B (PKB) (Castellano & Downward, 2011). Akt regulates cellular survival and metabolic processes via several downstream targets (Yang et al., 2004). The PI3K/Akt pathway was found deregulated in 45% of HGS-EOC cases (TCGA, 2011).

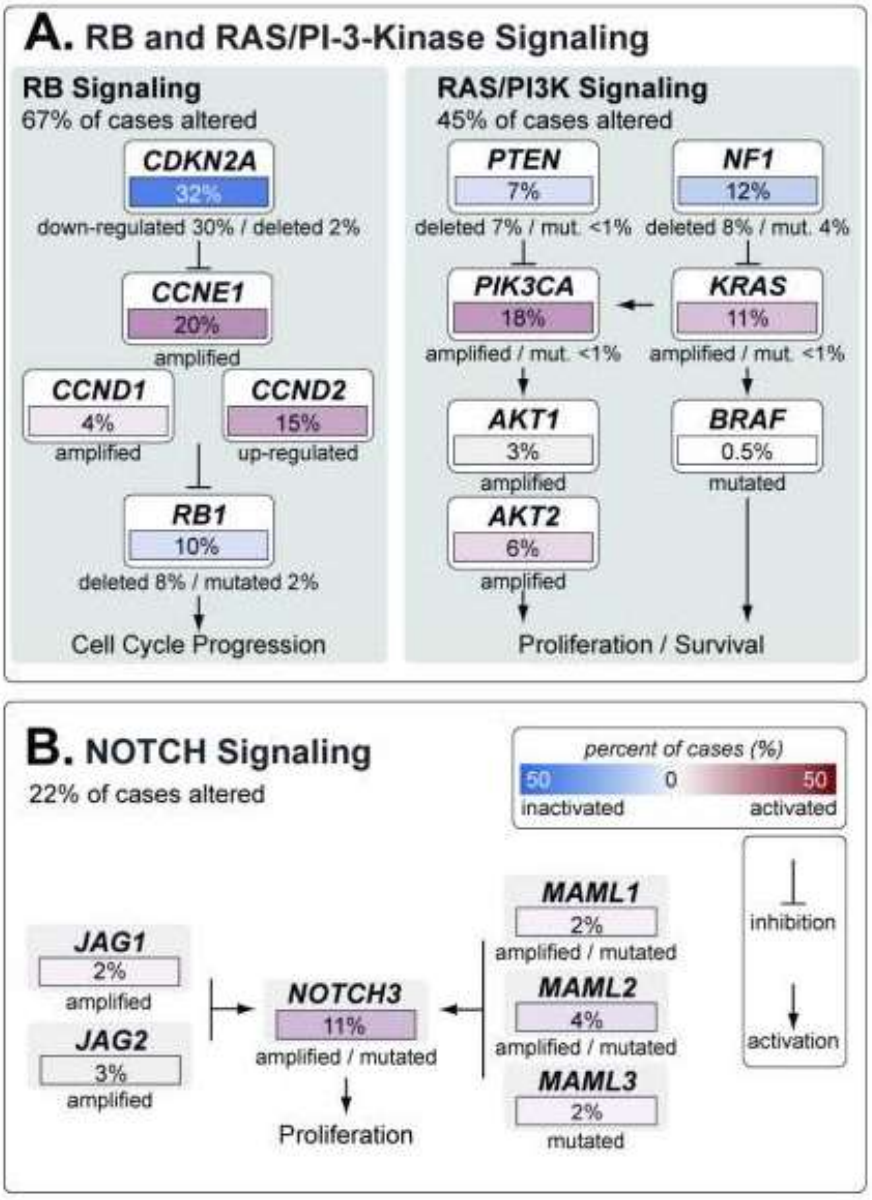
Notch pathway has a central role in carcinogenesis and was affected in 23% of cases (TCGA, 2011). Notch is a membrane-bound receptor that, after ligand interaction, undergoes two proteolytic cleavage by ADAM metalloprotease and γ-secretase. The released intracytoplasmic Notch domain (NICD) translocates to nucleus where activates the transcription of target genes (Kopan & Ilagan, 2009). In cancer, the constitutive Notch activation enhances the transcription of genes involved in cancer cell proliferation and invasion (Wang, Li, & Sarkar, 2010).

FOXM1 pathway deregulation was found in 87% of HGS-EOC cases. It is strictly correlated to p53 function. In case of DNA damage, p53 inhibits FOXM1 activity, avoiding the transcription of FOXM1-proliferation-related target genes (TCGA, 2011). In HGS-EOC the mutant p53 lost the inhibitory activity on FOXM1, thereby FOXM1-related gene transcription is aberrantly activated (Barger et al., 2015).

Mutations in BRCA1/2 were found in 22% of HGS-EOC cases due to a combination of somatic and germline mutations (TCGA, 2011). BRCA1/2 mutations are associated with the majority of HGS-EOC hereditary cases (Alsop et al., 2012). The cells with mutated BRCA1/2 show defects in homologous recombination (HR) DNA repair. Genomic alterations in HR genes, including also

ATM/ATR (2%) and PTEN (7%), render the cells sensitive to PARP inhibitors, the new drugs for OC treatment that will be discussed in the "Ovarian cancer therapy" section.

Figure 2 shows the molecular pathways altered in HGS-EOC.



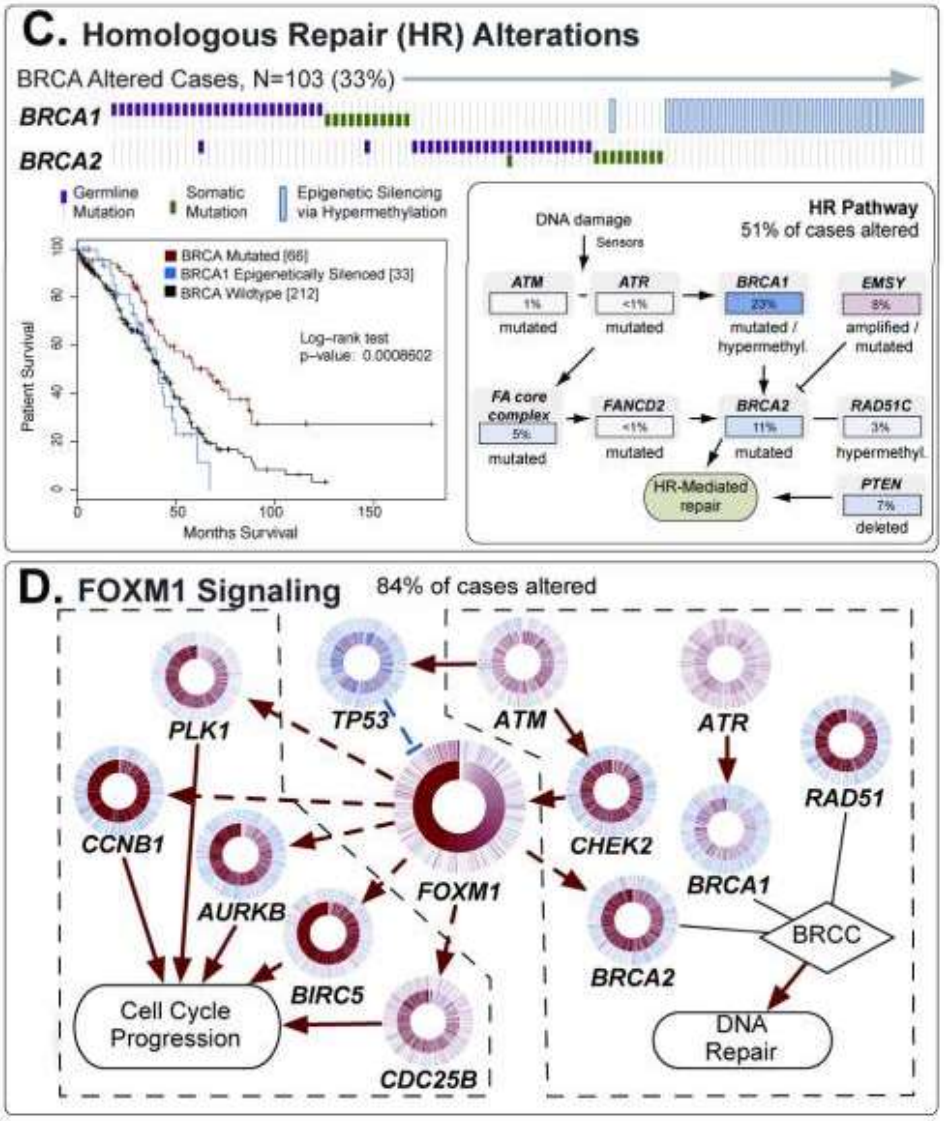


Figure 2. *Altered molecular pathways in high-grade serous epithelial ovarian cancer.* A) Rb signalling. CDKN2A: cyclin-dependent kinase Inhibitor 2A gene; CCNE1: cyclin E1 gene; CCND1: cyclin D1 gene; CCND2: cyclin D2 gene. Alteration frequencies are in percentage of all cases; activated genes are red, inactivated genes are blue. B) Ras/PI3K signalling. PI3CA: PI3K gene, AKT1: isoform 1 AKT; AKT2: isoform 2 AKT; NF1: neurofibromin gene; BRAF: B-Raf gene. Activated genes are red, inactivated genes are blue. C) Homologous Repair Alteration. On the top of figure, the gene alterations; on the left, the survival analysis of BRCA status; on the right the molecular pathway: ATM: Ataxia Telangiectasia Mutated gene; ATR: Serine/Threonine-Protein Kinase ATR; EMSY: BRCA2-Interacting Transcriptional Repressor EMSY gene. D) FOXM1 signaling. PLK1: polo-like kinase 1 gene; CCNB1: cyclin B1 gene; AURKB: Aurora Kinase B gene; BIRC5: Apoptosis Inhibitor Survivin gene; CDC25B: Cell Division Cycle 25B gene; CHEK2: checkpoint kinase 2 gene; RAD51: RAD51 Recombinase gene. Each gene is depicted as a multi-ring circle in which its copy number (outer ring) and gene expression (inner ring) are plotted such that each "spoke" in the ring represents a single patient sample, with samples sorted in increasing order of FOXM1 expression. The red arrows and the blue lines respectively represent excitatory and inhibitory interactions. Dashed lines indicate transcriptional regulation. (TCGA, 2011).

1.1.3. Ovarian cancer stages and therapy

About 70% of OCs are diagnosed at advanced stages due to the absence of early symptoms and efficacious screening methods (American Cancer Society).

Depending on the spread of the tumor cells to other organs, OCs are classified from stage I to IV.
The tumors confined to the ovaries or Fallopian tubes are classified as stage I; the tumors at stage II infiltrate the other organs (the uterus, the bladder, the sigmoid colon, or the rectum), but not the lynph nodes; OCs at stage III spread in the peritoneal cavity and into organs outside the pelvis; the most aggressive OCs are classified as stage IV: the cancer cells are found in the fluid around the lungs or inside the spleen,liver, lymph nodes and/or to other organs or tissues outside the peritoneal cavity (lungs and bones) (American Cancer Society).

The first step for treatment of OC is the surgery to remove the tumoral mass that often affects the uterus, both fallopian tubes, and both ovaries (hysterectomy with bilateral salpingo-oophorectomy). Depending on tumor stage, the surgery is followed by the chemotherapeutic treatmtent.
For stage I, most patients do not need any treatment after surgery.
For stage II and III the chemotherapeutic regimen consists in the intravenous platinum/taxane injection every 21 days, for six cycles (first-line chemotherapy).
Stage IV OCs are very difficult to eradicate. Often the surgery is preceeded by 3 cycles of chemoteraphy and followed by the first-line treatment (Basta et al., 2016).

Unlikely, almost all the patients relapse (low efficacy) manifesting severe drug reactions (high toxicity) and/or develop platinum resistance (Luvero, Milani, & Ledermann, 2014).
Based on the time to relapse and sensitivity to platinum, OC are classified as:

- platinum refractory (tumor progresses during first-line treatment);
- platinum resistant (recurrence within 6 months after completion of first-line treatment);
- partially sensitive (recurrence within 6–12 months);
- highly sensitive (recurrence after more than 12 months).

To define the recurrence, the Cancer antigen 125 (CA-125) is used as serum tumor marker. CA-125 is a protein secreted by OC cells into blood. The serous CA-125 levels were found elevated in 85% of patients with advanced OC (Luvero et al., 2014).
Platinum resistance is overcome by the addition of pegylated liposomal doxorubicin (PLD), topotecan, gemcitabine or targeted drugs to platinum treatment (Luvero et al., 2014).

The association of platinum/taxane with targeted drugs that inhibit angiogenesis has some clinical benefits in platinum-sensitive relapse (Luvero et al., 2014). Bevacizumab (a monoclonal antibody directed against the circulating vascular endothelial growth factor) or vascular endothelial growth factor receptor (VEGFR) inhibitors are the main antiangiogenic agents used (Luvero et al., 2014).

Recently, a group of inhibitors of the enzyme poly ADP ribose polymerase (PARP) have been shown to be efficacious in patients with germ-line mutation in BRCA genes at advanced stages.
PARP is an enzyme important for repairing single-strand breaks via the escission-base repair pathway.
The PARP inhibitor mechanism of action is based on synthetic lethality: the drug blocks PARP function, thus cancer cells fail to repair DNA single-strand breaks and accumulate DNA double-strand breaks. When BRCA proteins are not mutated, they permit to repair such DNA double-strand damage via homologous recombination (HRD) pathway. When BRCA genes are mutated, as in the majority of OCs, the cancer cells lost the capability to repair the DNA double-strand breaks and die due to the excessive DNA damage accumulation (Luvero et al., 2014).

Folate receptor alpha was found overexpressed in 80% of recurrent OCs. However, a therapeutic strategy with folate receptor inhibitors have not shown improvement in the overall survival (Kalli et al., 2008).

The last strategy suggested for recurrent OC consist in cancer immunotherapy in order to enhance the patient immune system to eliminate tumor cells. Among the several approaches, the most promising drugs are the immune checkpoint inhibitors, alone or in combination with other therapies and drugs.
In detail, cancer cells evade immne destruction by expressing on their cellular surface the programmed cell death ligand 1 (PDL-1) protein. PDL-1 binds to PDL receptor on CD8 T cells avoinding their activation. As a consequence, CD8 T cell-dependent cytotoxicity is inhibited and cancer cells survive (Juneja et al., 2017). The immune-checkpoint inhibitors bind to PDL-1 on cancer cells or to PD-1 receptor on CD8 T cells to avoid PDL-1/PD-1 receptor interaction and in turn to restore CD8 T cell antitmoral activity.
The different immunotherapeutic approaches for OC treatment are reviewed in Drerup et al. work (Drerup et al., 2015).

Nevertheless, all these new drugs or strategies were not shown to cure OC, but they just lead to the delay of tumor progression with little improvement in overall survival. For these reasons, new therapeutic approaches for OC are still needed.

1.2. PIN1

1.2.1. Structure and function

PIN1 was identified in 1996 in *Aspergillus nidulans* as a protein of approximately 18 kDa interacting with never in mitosis gene A (NIMA), a kinase essential for mitosis progression (Ping Lu, Hanes, & Hunter, 1996).

PIN1 belongs to the peptidylprolyl isomerase (PPIase) family of enzymes that function as accelerating agents, speeding up the *cis-trans* conformational switch in specific substrates.

On the basis of the affinity to immunosuppressive drugs cyclosporin A (CsA) and FK506, PPIase family consists in three different classes: the CsA-binding cyclophilins, the FK506-binding proteins (FKBPs) and the Parvulin-like PPIase which do not bind immunosuppressant.

PIN1 belongs to the last subclass and, in contrast to other isomerases, it is the unique that binds specific phosphorylated Ser/Thr-Pro motif in certain proteins to induce the conformational switch (**Figure 3**). For all the aminoacids the *trans* conformation is energetically more favourable than *cis*, but for proline both structures are closer in free energy so that X-Pro (where X is any residue) *cis* isomers appear with a frequency of 5-6% in proteins (Lu, Finn, Lee, & Nicholson, 2007).

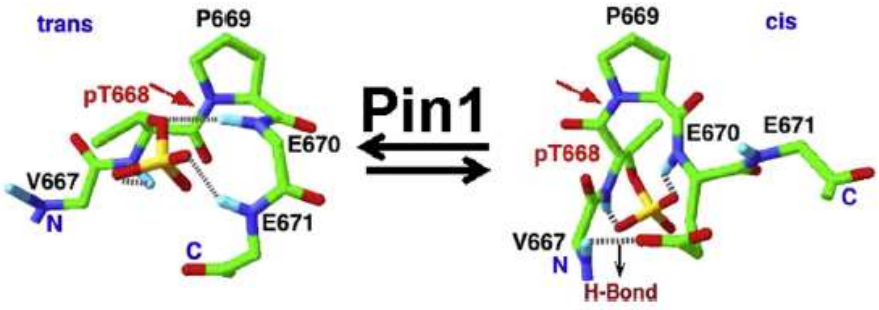


Figure 3. *PIN1-mediated cis/trans conformational switch* (K. P. Lu & Zhou, 2007).

Structurally, human PIN1 consists in two domains:

- the amino-terminal WW domain (referring to two invariant Trp residues), spans the first 39 residues folded into a three stranded β-sheet structure; within the WW domain exist two loops (from residue 16 to 21 and from residue 27 to 30).
- the carboxy-terminal PPIase domain, from the residue 50 to 163, is responsible of substrate recognition and catalytic activity (64-80 residues); the K63, R68 and R69 residues create a

positive charged phosphate-binding loop to facilitate binding to the pSer/Thr-Pro motif (Lee & Liou, 2018).

The WW domain and PPIase domain are connected by a flexible linker that spans from the residue 35 to 53 (Lee & Liou, 2018).

Figure 4 shows a representation of PIN1 structure.

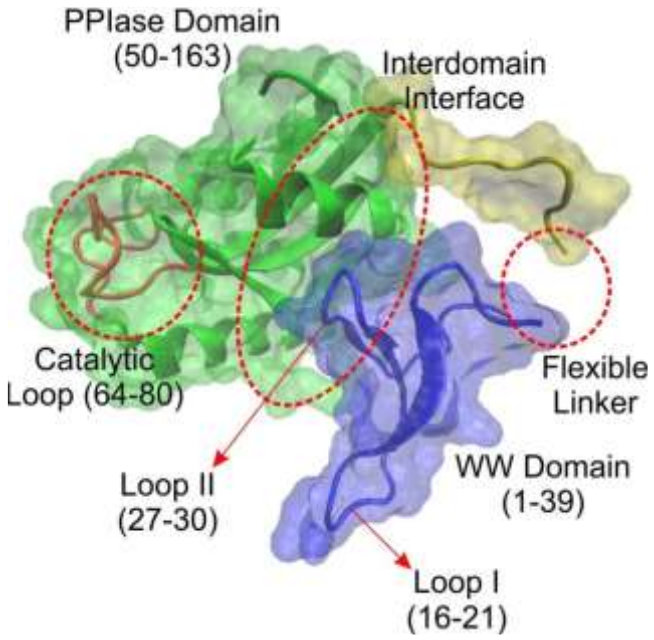


Figure 4. *PIN1 structure* (Lee & Liou, 2018).

The PPIase-catalyzed isomerization is an important regulatory mechanism in human physiology and pathology: the conformational change regulates various protein functions, including the catalytic activity, the phosphorylation status, protein interaction, subcellular location, and/or protein stability (Ping Lu et al., 1996). PIN1 substrates include proteins of many cellular processes such as the folding of newly synthesised proteins, immune-response, stress-response, neuronal differentiation and cell cycle control (Ping Lu et al., 1996) (Göthel & Marahiel, 1999) (Shaw, 2002).

1.2.2. PIN1 regulation of the expression

PIN1 expression is tightly regulated at multiple levels under physiological conditions, but it was found aberrantly activated or overexpressed in many human cancer, including prostate, breast, ovarian, cervical, brain, lung and melanoma tumors as well as in other pathologies such as

Alzehimer's disease, some cardiovascular dysfunctions, metabolic diseases, osteoporosis and diabetes (Bao et al., 2004) (Nakatsu et al., 2016).

PIN1 expression is regulated both at transcriptional and post-translational level.
The E2F (Ryo et al., 2002) or Notch (Rustighi et al., 2009) transcription factors might directly activate PIN1 promoter.

By contrast, the tumor suppressor gene BRCA1 could suppress PIN1 transcription (MacLachlan et al., 2000). The inhibitory BRCA1 function is lost in BRCA1-mutated OCs, thus PIN1 overexpression drives tumor progression (Steger et al., 2013).

Recently, micro-RNAs (miRNAs) have been identified as regulators of PIN1 expression. For instance, miR200c binds to a conserved region in the 3′-untranslated region (UTR) of PIN1 mRNA and prevents its translation (Luo et al., 2014). Mutations in this region of PIN1 can prevent the repressive effects of miR200c (Luo et al., 2014). The miRNA-200b and miR-296-5p also bind the 3′ UTR of PIN1 mRNA and down-regulate its expression. In cancer cells, both these miRNAs were found to be underexpressed, allowing PIN1 to sustain tumor progression (Zhang, Zhang, Gao, Wang, & Liu, 2013) (Lee et al., 2014).

PIN1 activity can also be regulated at post-translational level. There is evidence of post-translational modification by phosphorylation, SUMOylation and oxidization at specific sites and, more recently, of dimerization.

The S16 phosphorylation in WW domain by protein kinase A (PKA), 90 kDa ribosomal protein S6 kinase 2 (RSK2) or Aurora kinase A (AURKA) is sufficient to abolish PIN1 enzymatic activity. In cancer tissues PIN1 S16 results dephosphorylated thus PIN1 is active (Lu, Zhou, Liou, Noel, & Lu, 2002) (Cho et al., 2012) (Lee et al., 2013)

The phosphorylation of S71 in the PPIase domain enhances the activity of PIN1.
The death associated protein kinase 1 (DAPK1) is a tumor suppressor down-regulated in many cancer types that promotes cancer progression and metastasis. DAPK1 directly binds and phosphorylates S71 of PIN1. As a consequence, PIN1 is inactivated and its nuclear location is inhibited. Moreover, DAPK1 inhibits the ability of Pin1 to induce centrosome amplification and cell transformation (Lee et al., 2011).

The mixed-lineage kinase 3 (MLK3), a novel member of MAP3K superfamily, increases PIN1's catalytic activity by phosphorylating S138 in PIN1 PPIase domain (Rangasamy et al., 2012).

Similarly, S65 in PIN1's catalytic domain is the major phosphorylation site for Polo-like Kinase 1 (PLK1), a serine/threonine kinase overexpressed in many cancers (Eckerdt et al., 2005).

Recent studies suggest a novel mechanism that abolishes PIN1's enzymatic activity and oncogenic functions: the SUMOylation. It consists in the conjunction of a small ubiquitin-like modifier (SUMO) peptide on a lysine residue. The K6 in WW domain and the K63 in PPIase domain are the two SUMOylation sites identified in PIN1.
The SUMO1/sentrin specific peptidase 1 (SENP1) may remove the SUMO peptide on K63 restoring PIN1's activity. SENP1 overexpression increases the levels of deSUMOylated PIN1 and in turn the ability of PIN1 to induce centrosome amplification and cell transformation (Chen et al., 2013).

Under conditions of oxidative stress, PIN1 is oxidized on C113 in the catalytic site, inhibiting its enzymatic activity (Chen et al., 2015).

In **Figure 5** the structure of PIN1 with the regulatory post-translational modification sites are schematized.

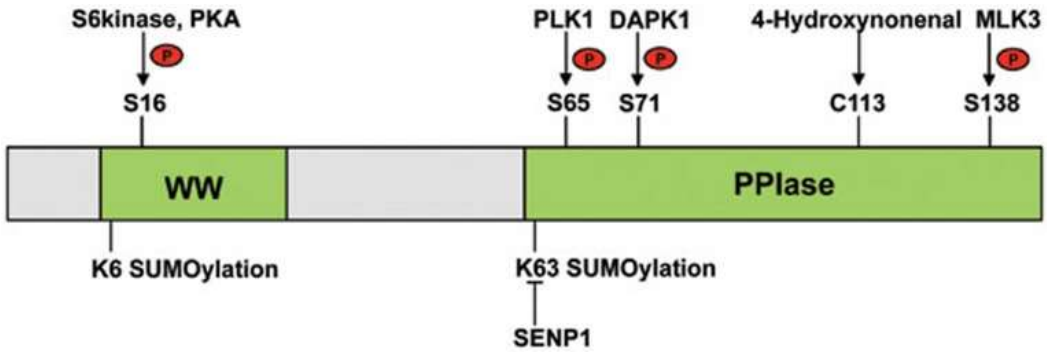


Figure 5. *Schematic representation of PIN1 and the regulatory post-translational modification sites* (Lu & Hunter, 2014).

The dimerization is another common post-translational process by which the proteins regulate their activation/inactivation. Recently, in the Lu's patent, it has been reported that the acetylation of PIN1

K46 residue and the formation of water-mediated hydrogen bonds between two different PIN1 protein monomers enhance the formation of the inactive PIN1 dimer. To support this finding, it has been demonstrated that the Q33K and/or E100D missense mutations in the WW domain may keep PIN1 in the constitutively active monomer and increase PIN1 tumorigenic activity (US20170204466A1, 2017)

1.2.3. PIN1 and cancer-driving molecular pathways

1.2.3.1. PIN1 targets in cancer

The neoplastic disease is a multistep process by which normal cells evolve progressively to a malignant phenotype by acquiring via distinct mechanisms and at various times characteristic functional capabilities that enable tumor growth and metastatic dissemination. Hanahan and Weinberg summarized these capabilities as eight hallmarks of cancer: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011). Each one is governed by alterations in the main molecular pathways including MAPK, PI3K/AKT, WNT/β-catenin, p53 and RB signaling (Vogelstein & Kinzler, 2004).

The increase of PIN1 level alone is not sufficient to induce fully transformation of normal cells, but it triggers some early events of tumorigenesis and tumor progression by regulating directly or indirectly more than 40 oncogenes/growth promoters and 20 tumor suppressors/growth inhibitors of such pathways (**Figure 6**) (Zhou & Lu, 2016).

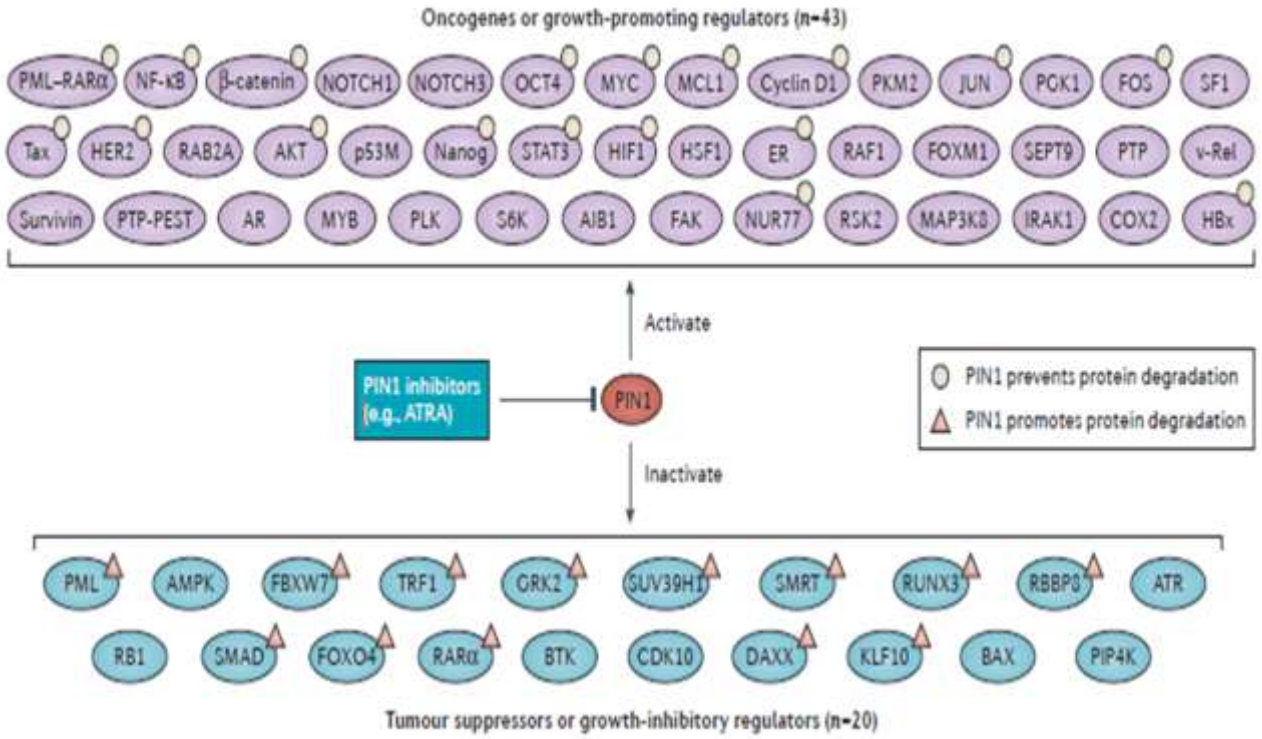


Figure 6. *Oncogenes/growth promoters and tumor suppressors/growth inhibitors regulated by PIN1.* The All-*trans* retinoic acid (ATRA) is a PIN1 inhibitor (Zhou & Lu, 2016).

Among them, PIN1 expression positively correlates with cyclin D1 levels that mediates aberrant cellular growth and proliferation (Liou, 2001). The strictly correlation between PIN1 and cyclin D1 expressions is confirmed by the evidence that PIN1 null mice show a phenotype similar to those of cyclin D1-deficient mouse: loss of body weight, retinal degeneration, mammary gland growth delay and testicular atrophy (Liou et al., 2002).

PIN1 regulates the expression of the cyclin D1 at both the transcriptional and post-translational levels. At the transcriptional level, PIN1 activates the transcription of gene encoding cyclin D1 (CCND1) via β-catenin (Ryo, Nakamura, Wulf, Liou, & Lu, 2001), c-Jun N-terminal Kinases (JNK), c-Jun and p65/RelA nuclear factor-κB (NF-κB) subunit.

PIN1/β-catenin interaction will be discussed in the following paragraph "PIN1 and WNT/β-catenin signaling".

In the Ras signaling pathway, the activation of a kinase cascade leads to phosphorylation and activation of JNK (c-Jun N-terminal kinase), which phosphorylates and activates the transcription

factor c-Jun. PIN1 can bind and isomerize both phosphorylated JNK and phosphorylated c-Jun to potentiate c-Jun transcriptional activity at the CCND1 promoter (Wulf et al., 2001).

Similarly, PIN1 is involved in NF-κB-dependent cyclin D1 expression. NF-κB is a heterodimeric complex of p65/RelA and p50, associated with the inhibitor IκBα. A variety of cytokines and growth factors promote IκB kinase complex (IKK)-dependent phosphorylation of IκBα, hence NF-κB activation. The phosphorylation at T254 in p65 is a key signal to PIN1 binding and isomerization. The conformational change ehnhances the p65 nuclear localization, stability and transactivation (Ryo et al., 2003).

At the protein level, PIN1 recognizes the phosphorylated T286 in cyclin D1. The isomerization of cyclin D1 leads to its stabilization and accumulation in the nucleus, where, in concert with other proteins, drives the cell cycle progression (Liou et al., 2002).

In addition to cyclin D1, other oncogenic proteins stabilized by PIN1 are Akt, pRb, myeloid cell leukemia 1 protein (MCL-1) and Stat3.
Deregulation of Akt and pRb pathways play crucial roles in cancer cell survival, migration and proliferation.

PIN1-mediated isomerization of Akt is critical for Akt signaling cascade activation that in turn activates cyclin D1, p53 and IKK-NFκB to enhance tumor progression (Liao et al., 2009).

PIN1 isomerization of pRb facilitates its binding to CDK–cyclin complexes in mid- to late G1. The hyperphosphorylated Rb regulates the expression of genes that mediate entry into the S phase via the E2F transcription factor. In cancer, PIN1 overexpression leads to pRb pathway iperactivation (Rizzolio et al., 2012) (Rizzolio et al., 2013).

Similarly, PIN1 causes MCL-1 conformational change to stabilize and support MCL-1 anti-apoptotic function. Briefly, MCL-1 is phosphorylated by GSK-3β, facilitating MCL-1 association with the E3 ligase β-TrCP. The interaction between MCL-1 and the GSK-3β–E3 ligase β-TrCP complex leads to MCL-1 ubiquitination and degradation (Ding et al., 2007). PIN1-mediated isomerization may prevent MCL-1 association with the GSK-3β–E3 ligase β-TrCP complex, blocking MCL-1 degradation, but further studies are required (Ding et al., 2008).

Finally, the transcription factor STAT3 is involved in inflammation-induced carcinogenesis and is constitutively activated in several cancers. PIN1 promotes the transactivation of STAT3 and in turn the transcription of the target genes, such as the antiapoptotic Bcl-xL (Lufei, Koh, Uchida, & Cao, 2007).

1.2.3.2. PIN1 and PI3K/AKT signaling

As discussed in the "Mutated genes and cancer-driving pathways in ovarian cancer" previous paragraph, the aberrant PI3K/Akt pathway activation drives OC development and progression. Amplification/overexpression of growth factor receptors (such as HER2/Neu or EGFR), somatic mutation of Ras oncogenes, mutation or amplification/overexpression of PI3K or loss of PTEN function result in PI3K signaling constitutive activation.

In human cancers, there is the evidence of a strictly correlations between the expression level of PIN1 and activated Akt (pS473-Akt), thereby it is suggested that PIN1 might play a pivotal role in OC. Moreover, in breast cancer patients, high pS473-Akt levels with high PIN1 expression levels correlate with poorer prognosis than either factor alone (Liao et al., 2009).

At molecular level, PIN1 binds to the phosphorylated T92 and T450 of Akt. The PIN1-mediated isomerization stabilizes Akt. As a result, the aberrant expression of the Akt downstream targets, including cyclin D1, p53 and IKK-NFκB, promotes cancer cell survival, migration and proliferation (Liao et al., 2009).

1.2.3.3. PIN1 and WNT/β-catenin signaling

Under pathological and some physiological conditions, β-catenin, the main effector of WNT signaling, is regulated by PIN1.

In normal and unstimulated cells, β-catenin associates with cadherin at plasma membrane and has a role in cell-to-cell adhesion. The regulatory adenomatous polyposis coli protein (APC)/Axin/glycogen synthase kinase-3 β (GSK-3β) complex binds cytosolic β-catenin leading to its ubiquitination and proteasomal degradation. The activation of the Wnt receptor complex (Frizzled receptors and low-density lipoprotein receptor-related proteins 5 and 6) by WNT proteins triggers the displacement of the multifunctional GSK-3β. As a consequence, β-catenin is stabilized and translocates to the nucleus, where it binds to T cell factor/lymphoid enhancer factor (LEF/TCF)

transcription factor, displacing co-repressors and recruiting additional co-activators to Wnt target genes (MacDonald, Tamai, & He, 2009). PIN1 avoids APC-β-catenin interaction resulting in β-catenin nuclear stabilization and activation of gene-related transcription (Ryo, Nakamura, Wulf, Liou, & Lu, 2001).

Among the genes regulated by β-catenin there are those necessary for the cellular program named "epithelial-to-mesenchymal transition" (EMT). Via the EMT, the epithelial cells lost the cell–cell adhesion and aquire the stem cell feautures, including the ability to physically disseminate from primary tumors, the chemo-resistance, the pluripotency and the self-renewal capabilities (Hanahan & Weinberg, 2011). Thereby, PIN1 indirectly sustains the EMT and amplifies the WNT/β-catenin signaling to maintain the malignat phenotype (Kim, Choi, Cho, Kim, & Kang, 2009).

1.2.3.4. PIN1 as molecular timer of cell cycle

PIN1 coordinates different events of G1/S and G2/M transitions of cell cycle acting as molecular timer of cell cycle.

PIN1 levels are high at G1/S transition in normal cells but become constitutively elevated in transformed cells resulting in high cyclin D1 expression and reduced cell cycle arrest factors, including p27 (Lin et al., 2015). In non-proliferating cells, p27 retards the cell cycle progression by inhibiting CDK2 kinase and in turn cyclin A and cyclin E activation. PIN1 binding to p27 in cancer cells leads to its dissociation from CDK2, resulting in higher cell proliferation. Paradoxically, PIN1 inhibits p27 degradation by proteasome (Cheng, Leong, Ng, Kwong, & Tse, 2017).

Moreover, PIN1 might directly interact with the tumor suppressor FOXO4, avoiding its translocation to nucleus and in turn inhibiting p27 activation of transcription and of the other cell cycle arrest genes (Lu & Hunter, 2014).

Among the proteins directly regulated by PIN1 at G1/S transition, β-catenin is the most important. As explained in "PIN1 and WNT/β-catenin signaling" paragraph, under WNT stimulation, PIN1-mediated isomerization leads to β-catenin accumulation in the nucleus and to the activation of the transcription of target genes including cyclin D1 and other cell cycle regulators (Ryo et al., 2001).

As the cell cycle proceeds, PIN1 is required to coordinates G2/M transition and mitotic progression. Indeed, PIN1 was originally identified in yeast as an isomerase that binds and suppresses the mitotic kinase NIMA avoiding the mitotic catastrophe (Ping Lu et al., 1996).

CDC25c and Aurora-A are just some of the several phosphoproteins that interact with PIN1 at G2/M transition. CDC25c is phosphorylated by at least two different kinases, Cdc2/cyclin B and Plk1, to allow the dissociation of a small peptide (Cdc25c). Cdc25c subsequently translocates into the nucleus, where it interacts with PIN1. PIN1-mediated conformational change triggers Cdc25c dephosphorylation by PP2A inhibiting entry into mitosis (Zhou et al., 2000) (Stukenberg & Kirschner, 2001).

Plk1 activation and Cdc25c phosphorylation is enhanced also by Aurora-A-Bora complex. The formation of the Aurora-A-Bora complex is permitted because Aurora-A phosphorylates on S16 and inactivates PIN1, thereby cell cycle might proceed to M phase. In cancer, the overexpressed PIN1 binds Bora promoting its degradation and avoiding the formation of the Aurora-A-Bora complex. As a result, PIN1 remains active and delays mitotic entry (Lee et al., 2013).

At G2/M transition, PIN1 might act directly on RNA polymerase II. It is known that mRNA synthesis is repressed during mitosis, which reflects RNA Polymerase II dissociation from active genes to allow chromosome condensation. PIN1 participates in these processes by stimulating CTD-RNA Polymerase II hyperphosphorylation by Cdc2/cyclinB responsible for transforming the RNA Polymerase II from S-phase to mitotic isoform (Xu & Manley, 2007).

Recently, PIN1 was found negatively regulated during M phase by anti-tubulin molecules, a class of anticancer drugs. The novel discovered Cdk- large tumor suppressor (LATS) proteins-PIN1 signalling axis controls the balance between cancer cell proliferation and death in the presence of anti-tubulin drugs. The anti-tubulin drugs induce LATS proteins phosphorylation by Cdk1, which in turn negatively regulate PIN1 and permit the activation of the apoptotic program. However, in the presence of high expression, PIN1 induces anti-tubulin drug resistance and inhibits LATS with a negative feedback mechanism. Therefore, targeting PIN1 might restore drug sensibility, mitosis arrest and apoptosis (Yeung, Khanal, Mehta, Trinkle-Mulcahy, & Yang, 2018).

1.2.3.5 PIN1 and apoptosis

Programmed cell death serves as natural barrier to cancer development, but cancer cells evolve a variety of strategies to circumvent it and promote proliferation (Hanahan & Weinberg, 2011). The apoptotic evasion represents the main cause of chemotherapeutic and radiotherapeutic resistance that

characterizes the aggressiveness of human malignant tumors. Several studies report the involvement of PIN1 in preventing the apoptotic process.

The majority of human cancers present mutations in the TP53 tumor suppressor gene that contribute to the malignant phenotype sustaining the migration, invasion and genomic instability of cancer cells. PIN1 has the ability to interact with and isomerize the mutant p53 in cancer cells preventing p53 association with the ubiquitin-ligase MDM2. The stabilized p53 associates with and inhibits the endogenous p63, a suppressor of metastasis, and Smad2, a pro-apoptotic protein enhancing tumor progression (Girardini et al., 2011).
Based on these evidences, PIN1 inhibition could be a beneficial therapeutic chance at later stage of cancer progression, but not at early stage when PIN1 seems to act as a tumor suppressor (Mantovani et al., 2007). As in normal cells, during the early stage of cancer, in the presence of WT p53 and low levels of the inhibitor of apoptosis-stimulating protein of p53 (iASPP), PIN1 supports the apoptotic function of p53 (Mantovani et al., 2007).

The activation/inactivation of the apoptotic program is an intricate process, which involve not only p53, but several proteins strictly regulated.
PIN1 might directly regulate the stability and localization of both pro-apoptotic proteins, such as death-associated protein Daxx and promyelotyc leukemia protein (PML), or anti-apoptotic proteins, such as MCL-1 and Survivin (Lu & Hunter, 2014).

Daxx is a Fas-interacting protein activated by UV damage, oxidative stress and glucose deprivation (Salomoni & Khelifi, 2006). Pin1 binds the S178 of Daxx and in turn, the conformational change facilitates Daxx degradation via the ubiquitin-proteasome pathway (Ryo et al., 2007).

In a similar way, PIN1 binds at four phosphorylated Serine residues in PML protein and enhances PML degradation in tumor cells (Reineke et al., 2008). PML is essential for the induction of both intrinsic and extrinsic apoptotic pathways and was found under-expressed in several cancers (Gurrieri et al., 2004).

PIN1 modulation influences the activity and stability of MCL-1 and Survivin.
PIN1-mediated isomerization of MCL-1 increased MCL-1 stabilization and its anti-apoptotic function (Ding et al., 2008), whereas PIN1-mediated isomerization of Survivin avoids pro-caspase 9

activation, the main effector of apoptotic program. Both Survivin and PIN1 are up-regulated in tumors and sustain cancer cell proliferation and chemoresistance (Cheng et al., 2013) (Dourlen et al., 2007). In addition, both MCL-1 and Survivin may be indirectly regualted by PIN1 via the activation of Notch1 transcriptional program that sustains tumor aggressiveness (Sorrentino, Comel, Mantovani, & Del Sal, 2014).

A scheme of PIN1 and its anti-apoptotic targets is shown in **Figure 7.**

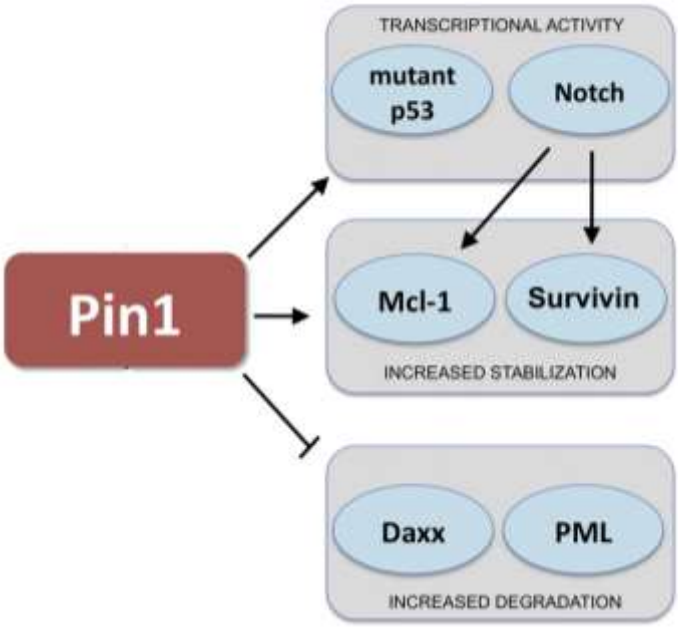


Figure 7. *PIN1 and its anti-apoptotic targets* (Sorrentino et al., 2014).

On the other hand, some treatments could cause indirectly PIN1 degradation and cancer cell death. For example, longer Taxol treatment induces microtubule damaging, G2/M arrest and phosphorylation of Bcl-2. Such modification promotes Bcl-2 translocation into the nucleus, where it interacts with PIN1. The interaction with Bcl-2 promotes PIN1 proteasomal degradation. As PIN1 level decreases, Bcl-2 is dephosphorylated to its native form. However, the Bcl-2 anti-apoptotic function is inhibited due to Bcl-2 inability to relocate to mitochondria, thus the cells continue to die by apoptosis (Basu et al., 2002).

Despite the strong evidences that describe PIN1 as a negative regulator of apoptosis and tumor enhancer, accumulating works report PIN1 pro-apoptotic function at different levels and steps of the apoptotic pathways. PIN1 is indeed considered a context-dependent signal transducer since it acts as

anti- or pro-apoptotic agent depending on its interactors and environmental cues as discussed in the following “PIN1 as conditional tumor suppressor” paragraph.

1.2.4. PIN1 as conditional tumor suppressor

In the light of the above, PIN1 has a pivotal role in cancer progression, but Yeh and Means described PIN1 as a “conditional” tumor suppressor since, depending on tumor cell context, it might play an anti-cancer function (Yeh & Means, 2007).
Several studies support this hypothesis showing that PIN1 destabilizes many oncoproteins (c-Myc, cyclin E and others) and regulates various aspects of mitochondrial apoptosis.

The activation of Ras leads to phosphorylation of c-Myc at S62 by ERK during early G1 phase. Such modification stabilizes Myc, which accumulates at high levels and allows cancer cell proliferation. When Ras activity declines after cessation of growth stimuli, GSK-3β is activated and phosphorylates c-Myc on T58. This phosphorylation is important for PIN1 recognition and to facilitate c-Myc dephosphorylation at S62 by PP2A, which then promotes c-Myc turnover by ubiquitin-proteasome pathway (Yeh et al., 2004).

The regulation of cyclin E is remarkably similar to that of c-Myc. Cyclin E is important for the progression of cells into S phase and its activity is modulated by phosphorylation of S384 by Cdk2 and of T380 by GSK-3β, which are respectively equivalent to S62 and T58 phosphorylation in c-Myc. Therefore, PIN1 binding to cyclin E is dependent on phosphorylation of S384 and promotes its ubiquitination and degradation via proteasome. In the absence of PIN1, cyclin E is stabilized and, in combination with other protein alterations, leads to cell cycle defects and genomic instability (Yeh, Lew, & Means, 2006).

PIN1 can be considered a master regulator of mitochondrial apoptosis (Sorrentino et al., 2014). In particular, when p53 is functional and not mutated, PIN1 has a central role in the regulation of its stability and transcriptional activity to support cancer cell death. Three sites, S46, S33 and S315, are critical for PIN1 binding and protection of p53 from proteolytic cleavage. The conformational change in the protein facilitates additional modifications and the recruitment of other factors resulting in p53 loading on target promoters and functional activation (Zacchi et al., 2002).

PIN1 can favor apoptosis by directly acting on inhibitors of p53 transcriptional activity such as iASPP or Che-1 protein.
iASPP is overexpressed in several tumors and binds the proline-rich domain (PRD) of p53. PIN1 overexpression might prevent this association and enhance p53 pro-apoptotic function (Mantovani et al., 2007). Moreover, PIN1 triggers the degradation of Che-1 protein via MDM2 (De Nicola et al., 2007).

In addition to the transcription-dependent function of p53, many evidences establish PIN1 suppressive role also in transcription-independent p53 activity. Stress conditions induce the phosphorylation of p53 on S46-P motif by the pro-apoptotic homeodomain interacting protein kinase 2 (HIPK2). PIN1 recongnizes such phosphorylated residues and isomerizes p53 that in turn translocates to mitochondria. The mitochondrial accumulation amplifies the apoptotic response as demonstrated by transcriptional activation of pro-apoptotic target genes (Sorrentino et al., 2013) (Wulf, Liou, Ryo, Lee, & Lu, 2002). In addition, PIN1 might directly isomerize and stabilize HIPK2. As a consequence, HIPK2 accumulates and phosphorylates p53 on S46 (D'Orazi et al., 2002) (Bitomsky et al., 2013).

Follis et al. provide another explanation for the interplay between p53 and PIN1 to promote apoptosis. Indeed, PIN1-catalyzed isomerization of P47 allows p53 interaction with distinct region of BAX and possibly formation of transient BAX/p53/PIN1 ternary complex. Within the complex PIN1 enhances p53-dependent BAX activation, hence pro-apoptotic signaling. Accordingly, silencing of either PIN1 or BAX reduces apoptosis in wild-type p53 cells (Follis et al., 2015)
Compatible with the hypothesis that PIN1 may have tumor suppressive function is the evidence that it results under-expressed in some p53 wild-type tumors (Teng, Hacker, Chen, Means, & Rathmell, 2011). For example, PIN1 level is low in human clear cell renal carcinoma, in part due to deletion of PIN1 locus. The genetic loss of function is an hallmark of tumor suppressors, thus PIN1 restoration might produce reduction in tumor cells growth (Teng et al., 2011).

In cells lacking p53, the sibling p73 might induce apoptosis in response to both E2F overexpression and chemotherapeutic treatments, providing a strong barrier to tumorigenesis. PIN1 contributes to full activation of p73 promoting its interaction with the acetyl-transferase p300 and its recruitment on apoptotic target promoters (Mantovani et al., 2004).

Another pro-apoptotic role of Pin1 is associated to the generation of radical oxygen species (ROS) that is very high in cancer cells. Under oxidative stress conditions, PIN1 interacts with and isomerizes the growth factor adapter $p66^{Shc}$ enhancing its translocation to mitochondria. Within the mitochondria, $p66^{Shc}$ acts as an oxidoreductase and generates ROS. ROS accumulation leads to organelle dysfunction and cell death (Pinton et al., 2007).

PIN1 might have a pro-apoptotic role by acting directly on the pro-survival protein ATR. ATR contains a BH3-like domain that allows ATR-Bid interaction at mitochondria, suppressing the pro-apoptotic Bax protein recruitment, cytochrome c release and apoptosis. PIN1-mediated isomerization of ATR inhibits the mitochondrial activity of ATR enhancing cell death. In response to UV damage, PIN1 is inactivated via DAPK1 and in turn the *cis*-isomeric ATR is stabilized and sustains cell survival (Hilton et al., 2015).

In **Figure 8** are represented the main PIN1 pro-apoptotic targets discussed.

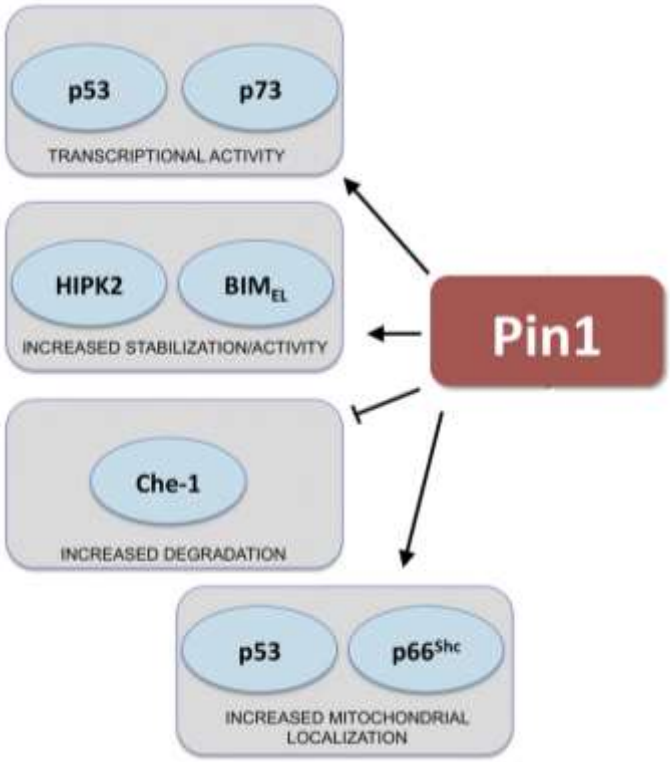


Figure 8. *PIN1 and its pro-apoptotic targets.* $BIM_{EL}$is BH3-only family member that has pro-apoptotic activity in neurons (Sorrentino et al., 2014).

1.2.5. PIN1 as a possible biomarker in cancer

In prostate cancer, a higher probability of recurrence after prostatectomy was observed in patients with high PIN1 level compared with patients with low PIN1 level. These results suggest that PIN1 expression might be a prognostic marker and/or a novel therapeutic target in prostate cancer (Ayala et al., 2003).

New biomarkers are necessary to be defined for stage I lung adenocarcinoma since, to date, few are available to predict prognosis. A study of 15 patients with surgically resected stage I lung adenocarcinoma identified 12 up-regulated proteins, including PIN1, that might be accepted as new biomarker (Ha et al., 2013).

PIN1 was found highly expressed also in many primary and metastatic breast cancer tissues, including Her2+, ERα+ and basal-like breast cancers (Wang, Liu, & Zhang, 2015). PIN1 levels in breast cancer tissues positively correlate with cell transformation and proliferation, tumor stage, metastasis, angiogenesis, drug sensitivity, recurrence and survival rate (Wang et al., 2015). Therefore, PIN1 expression was associated with poorer outcome and may be a new biomarker for breast cancer (Wang et al., 2015).

In papillary thyroid carcinoma, PIN1 was found to be differentially expressed in diverse specimens, but at advanced stage it was found strictly up-regulated (Jiang, Chu, & Zheng, 2016).

Similar evidence was found in non-small cell lung cancer (NSCLC) patients, in which PIN1 level was considered an independent prognostic factor and was correlated with poor survival (He et al., 2007).

All these studies suggest that PIN1 may be used to predict a patient's prognosis and to develop novel therapeutic strategies for individual treatment.

1.2.6. PIN1 inhibitors

In the last ten years, several work groups have tried to develop efficient PIN1 inhibitors with both covalent and non-covalent binding mechanisms.

In 2009, Guo and co-workers started for Pfizer a PIN1 program in order to discover new PIN1 small molecule inhibitors. The first approach consisted in a high-throughput screening (HTS) of more than one million compounds, but it failed.

Then, on the basis of the similarity between the active sites of PIN1 and the peptidyl-prolyl *cis-trans* isomerase FKBP-12 , they developed a compound able to bind PIN1, but unfortunately unable to enter cells due to a poor permeability conferred by the phosphate group (Guo et al., 2009) .

In the following years, the same researchers tried to optimize this compound by modifying the functional groups within the molecule, but they didn't achieve great results.

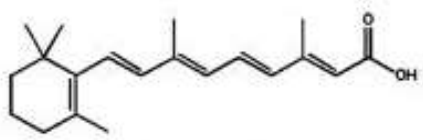
By contrast, Potter and co-workers from Vernalis reported imidazole derivative class of molecules with interesting PIN1 enzymatic activity (Potter et al., 2010).

During the same time, further design, supported by x-ray crystallographic structures, led to identification of new 3-chlorophenylimidazole acid derivatives bearing a PIN1 inhibition activity (Potter et al., 2010).

About four years later, Guo and co-workers revised their studies and synthesized different dihydrothiazole derivatives. These new compounds showed a nanomolar PIN1 inhibition activity and worked also in cancer cells (Guo et al., 2014).

In 2015, the *all-trans* retinoic acid (ATRA) was identified as a novel PIN1 inhibitor by applying on about 8200 compounds a mechanism-based HTS screening (**Figure 9A**). ATRA-PIN1 interactions consist in salt bridges between the carboxylic acid and PIN1 K63 and R69 residues, as well as hydrophobic interaction between aromatic moiety of ATRA and L122, M130, Q131 and F134 PIN1 residues (**Figure 9B**). This compound was approved for the treatment of acute promyelocytic leukemia (APL). ATRA interaction with PIN1 leads to the degradation of the protein encoded by the fusion oncogene PML-RARA responsible of APL. Furthermore, ATRA/PIN1 interaction inhibited triple-negative breast cancer cell growth in human cells and in animal models (Wei et al., 2015).

A)



All *trans* retinoic acid (ATRA)

B)

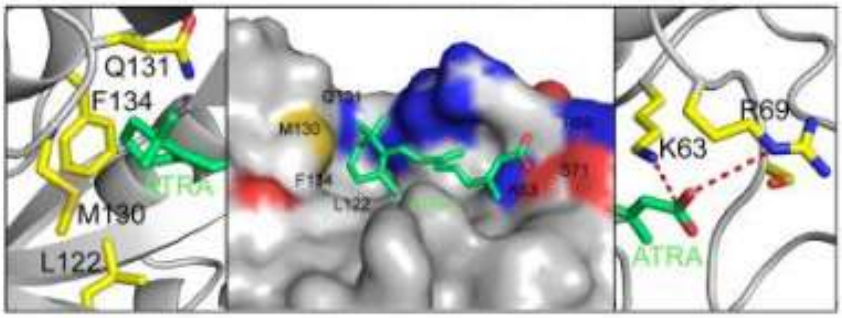


Figure 9. *All trans retinoic acid (ATRA) is a PIN1 inhibitor.* A) Structure of all *trans* retinoic acid (ATRA); B) ATRA binds PIN1(middle) via salt bridges between the carboxylic acid and K63 and R69 PIN1 residues (right), as well as via hydrophobic interaction between aromatic moiety of ATRA and L122, M130, Q131 and F134 residues (left) (Wei et al., 2015).

In following year, Xu and co-workers identified new thiazole derivatives containing an oxalic acid or an acetic acid. These compounds showed PIN1 inhibitory activity in the low micromolar range, but cellular assays were not reported (Zhao, Cui, Jin, Chen, & Xu, 2016).

By applying a novel HTS system, a chemical library of about 1000 compounds was screened and a selenium-containing comopund was identified as a PIN1 inhibitor. Then, by means of new modified selenium-containing derivatives and the analysis of the structure-activity relationships, Osada and co-workers identified a new PIN1 inhibitor able to inhibit the proliferation of the breast cancer MDA-MB-231 cells and to inhibit viability of induced cancer stem cell-like cells (Subedi et al., 2016).

In addition to the cited compounds, another class of molecules are the covalent PIN1 inhibitors.

Uchida and co-workers developed in 2011 a series of isothiazolone derivatives possessing a low PIN1 micromolar activity and also activity towards cyclophilin (Mori et al., 2011)

Very recently, Del Sal and co-workers, virtually screened a drug like collection of 200000 commercial compounds and tested the filtered compounds. As a results, they found a molecule that covalently binds PIN1 and that was able to impaire lung tumor growth (Campaner et al., 2017).

Unfortunately, all the cited compounds presented some limitations, therefore the development of a selective and efficient PIN1 inhibitor is still needed.

2. Rationale and aims

The PPIase PIN1 is a conserved enzyme, tightly regulated at multiple levels under physiological conditions, but aberrantly expressed in many pathologies (Bao et al., 2004) (Nakatsu et al., 2016).

The whole genome data released from The Cancer Genome Atlas (TCGA) consortium analyzed the presence of PIN1 alterations (amplification, deletion and mutation) in different tumor types, including OC. PIN1 was found mostly amplified in hormonal cancers and especially in HGS-EOC.

According to the genomic amplification, the PIN1 protein levels were found frequently increased in different types of cancers (Bao et al., 2004).
Experimental data revealed that PIN1-catalyzed isomerization of several key proteins enhanced the acquisition of hallmark capabilities of cancer by affecting diverse cell activities, including metabolism, mobility, cell cycle progression, proliferation, survival and apoptosis. Therefore, PIN1 expression correlates with poor prognosis suggesting that PIN1 migh be a good prognostic factor (Ayala et al., 2003) (Wang et al., 2015)
All these evidences suggest PIN1 as an attractive potential target for cancer therapy.

Based on this background and on the evidence that PIN1 involvment in OC is still poorly investigated, the first aim of my PhD project was to define PIN1 function in HGS-EOC, the most common and aggressive type of OC.

Since almost all the OC patients relapse due to severe toxicity of the treatments and/or platinum resistance development, new therapeutic chances for recurrent OC are needed.
Several PIN1 inhibitors have been developed in the last years, but all of them fail in anticancer activity in cells due to poor permeability of cell membrane (Guo et al., 2009)(Dong et al., 2010)(Guo et al., 2014).
These limitations necessitate the investigation of more potent and specific PIN1 inhibitors.

Once validated PIN1 as new oncogene in OC, the second aim of my PhD project was to develop an efficient PIN1 chemical inhibitor to give novel therapeutic chances for recurrent HGS-EOC and PIN1-overexpressing cancer patients.

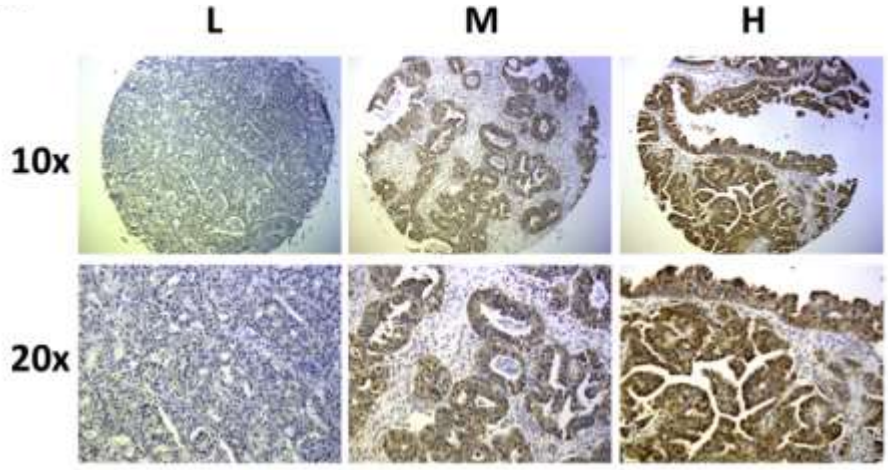
3. Results and discussion

3.1. PIN1 in ovarian cancer

3.1.1. PIN1 is overexpressed in serous ovarian cancer patients

We evaluated by immunochemistry the PIN1 expression in serous OC on tissue microarray (TMA). We analyzed 167 cases of serous OC and among them the 59.4% were grade 3. As shown in **Figure 10A**, the expression values were divided into two categories: low and medium-high. We found PIN1 significantly upregulated in tumor tissue compared to the adjacent normal tissue (13 cases) (pvalue 0.0012, Fisher exact test) (**Figure 10B**). In support of the results from TCGA, our data suggested that PIN1 was a potential therapeutic target in OC.

A)



B)

	Pin1		
	Low	Mid-High	Total
Serous	**77** (42.8%)	**90** (50%)	**167** (92.8%)
Normal	**12** (6.7%)	**1** (0.5%)	**13** (7.2%)
Total	**89** (49.4%)	**91** (50.6%)	**180** (100%)
p_value	**0.0012**		

Figure 10. *PIN1 is highly expressed in serous ovarian cancer*. A) Representative images at different magnifications of low (L), medium (M) and high (H) PIN1 expression in tumor tissues. B) PIN1 protein is upregulated in cancer vs normal tissues. Fifty percent of cancer tissues have medium-high expression of PIN1 compared to 0.5% in normal tissues (Russo Spena et al., 2018).

3.1.2. PIN1 down-regulation affects cancer cell viability

In order to evaluate if PIN1 affects cancer cell viability in human cells, KURAMOCHI, COV318, and OVCAR3 cell lines were KD (**Figure 11A**) and cell viability was analyzed for 6 days. PIN1 KD cells were less viable than control cells (**Figure 11B**). These data suggested that PIN1 is involved in the biological mechanisms that permit to cancer cells to survive and resist to cell death.

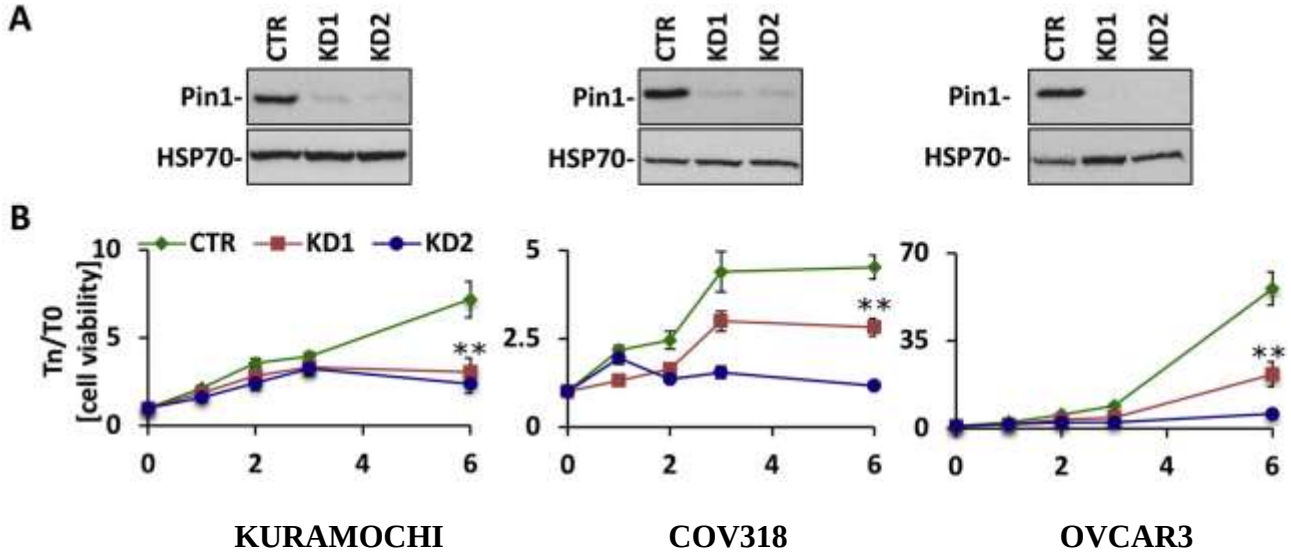


Figure 11. *Pin1 knock-down in ovarian cancer cells.* A) PIN1 expession was evaluated by Western Blot Analysis in OVCAR3, COV318 and KURAMOCHI cell lines. Images are representative of a single experiment. B) Cell viability was done in triplicates. X axis: days; values were normalized for T0; standard deviation values are showed; p-value < 0.05 (Russo Spena et al., 2018).

3.1.3. PIN1 down-regulation induces cancer cell death

Flow-citometry analysis using propidium iodide was performed to evaluate the population of sub-G1 cells. The propidium iodide is a fluorescent dye that binds to DNA by intercalating between the bases. The cells that are stained less intensely consist in cells ongoing death and are represented as a pick below the G1 peak (sub-G1). We found an increase in sub-G1 phase in PIN1 KD cells compared to control (two side t-test, p-value < 0.05) (**Figure 12A**).

To discriminate if a real apoptotic mechanism was activated, cells were analyzed for Annexin V staining by flow-citometry. The Annexin V marks the phosphatydilserine, a phospholipid of the cell membrane. In healty cells, the phosphatydilserine is located along the cytosolic side of plasma membrane but in apoptotic cells it traslocates to extracellular side and might be marked with Annexin

V. The KD cells showed an increased number of apoptotic cells (early and total apoptosis) compared to normal cells (two side ttest, p-value < 0.05), (**Figure 12B and 12C**).

To understand the molecular mechanism that leads to apoptosis, caspase 3/7 activity was evaluated.The caspases 3/7 are the final effectors of the apoptotic program. We found an incrase in the activity of these protease enzymes in KD cells (two side t-test, p-value < 0.05), confirming that PIN1 inhibition promotes cancer cell death via the apoptotic cascade activation (**Figure 12D**).

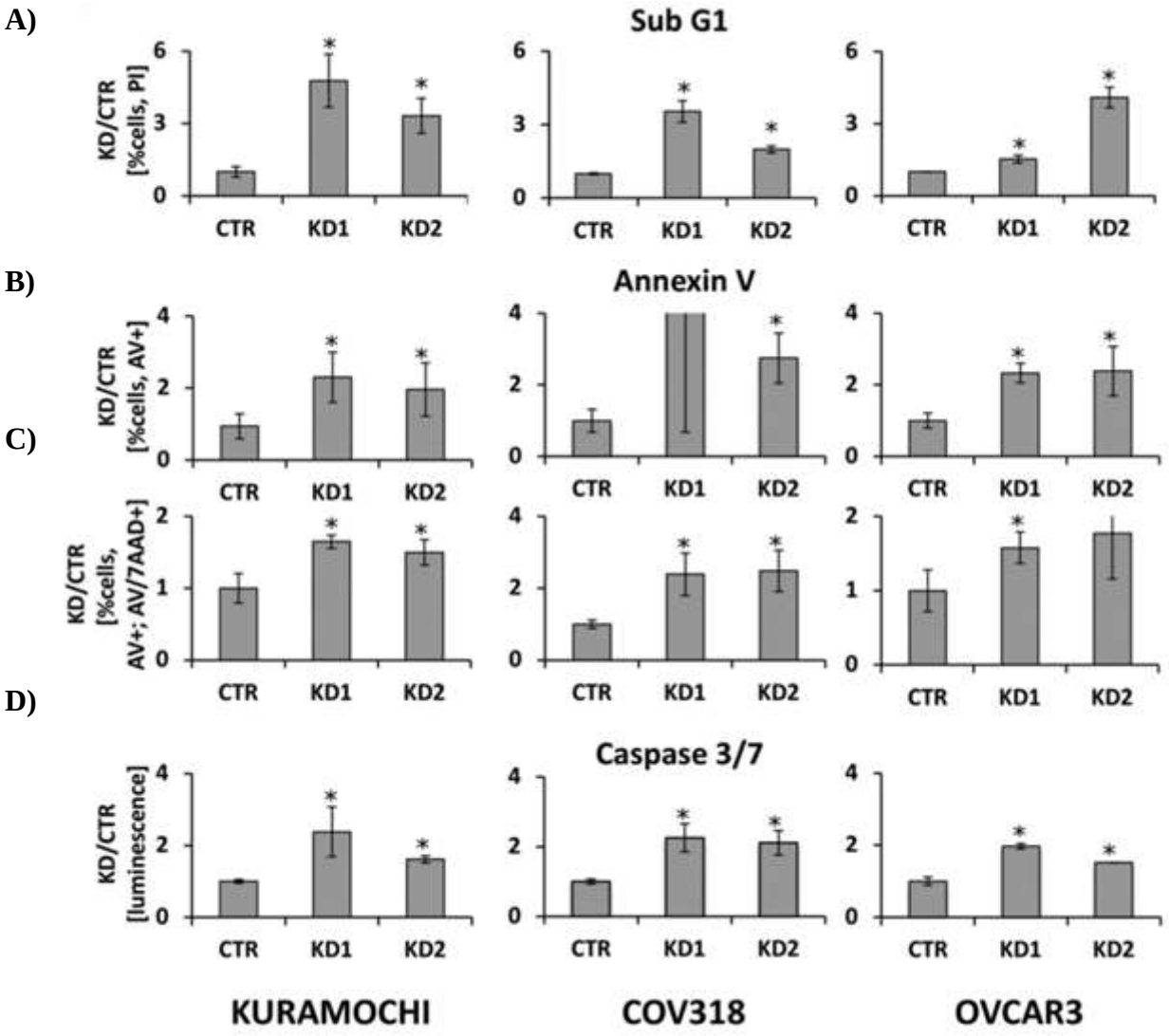


Figure 12. *PIN1 knock-down and apoptosis.* A) Sub-G1 was determined by propidium iodide staining (≥three independent experiments). B) Early and C) total apoptosis were determined by Annexin V/7-AAD staining (≥three independent experiments). D) Activation of caspase 3/7 was analyzed on cell extracts by luminescence assay (≥ two independent experiments). All the values on y-axis are normalized to the control. (*, p value < 0.05; standard deviation values are showed) (Russo Spena et al., 2018).

Taken toghether, our results gave the evidence that PIN1 is a new oncogene and a new therapeutic target in OC.

3.2. PIN1 chemical inhibitor

3.2.1. VS10 compound is a potential PIN1 inhibitor

As a first step to identify new PIN1 inhibitors, in collaboration with Prof. Tuccinardi of the University of Pisa, we tested the reliability of consensus docking in predicting the position of the ligand binding site for existing PIN1–ligand X-ray complexes. Ligands were extracted from their X-ray complexes. Such ligands were docked in all the structures by using ten docking procedures. A total of 12 ligand-protein structures were analyzed, with 1440 docking calculations. Reliability was assessed from the average root-mean-square deviation (RMSD) between the position of the ligand predicted by docking and their known position. The **Figure 13** shows that the docking procedures had an aRMSD in the range of 3.7-4.9 Å. AutoDock4 had the best result (smallest deviation)**.**

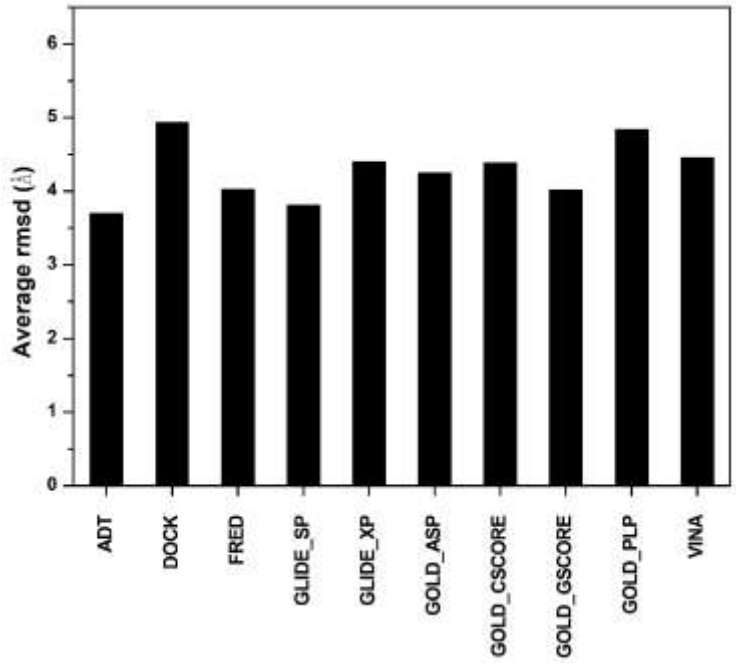


Figure 13. *Average root-mean-square deviations (RMSDs) for ten cross-docking procedures.* ADT: AutoDock; CSCORE: ChemScore; GSCORE: GoldScore; Vina: AutoDock Vina.

Then, the results of each docking procedure (data for each ligand docked into each PIN1 binding site) were clustered to search for common binding modes. As the consensus level increased from 2 (i.e.,

taking into account all the ligand−protein combinations that showed at least two out of ten docking poses clustered together) to the maximum value of 10, the average RMSD decreased from 3.7 Å to 0.7 Å (**Figure 14**). The best reliability achieved with consensus docking (0.7 Å at consensus 10) is about 5-times better than that obtained by using the best docking procedure in the cross-docking analysis (3.7 Å with AutoDock). However, as the consensus level increased, the percentage of all ligand−protein combinations retained ("survived") decreased, from 99% at a consensus level of 2 to 5% at a consensus of 10. These results mean that the quality of docking predictions increases with the consensus level, and that consensus docking improves the prediction of the ligand docking pose.

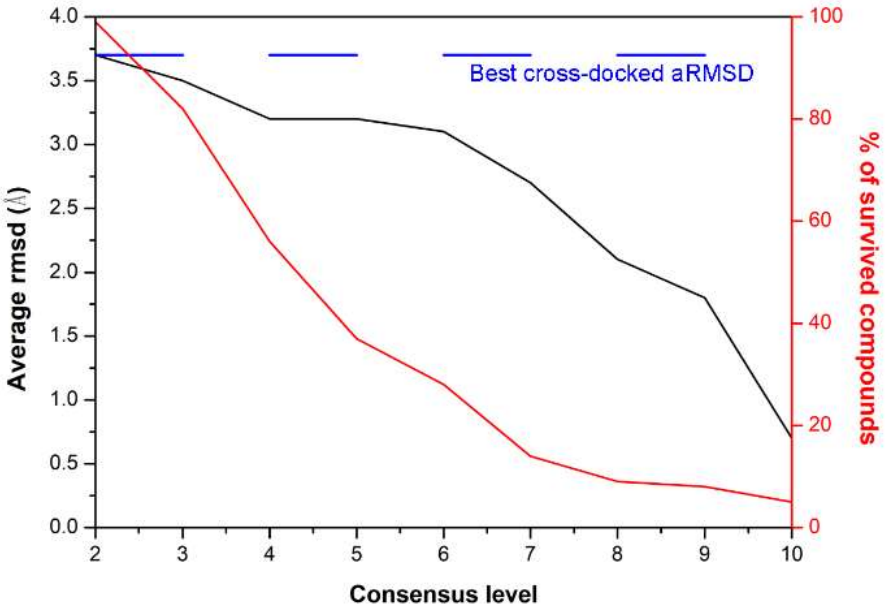
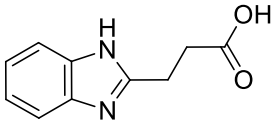
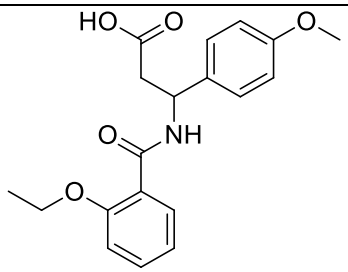
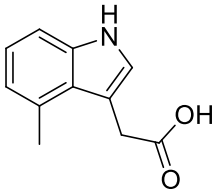
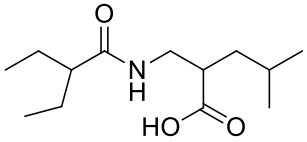
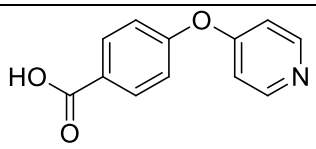
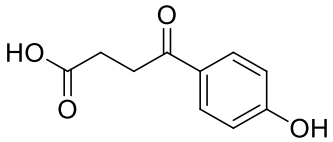
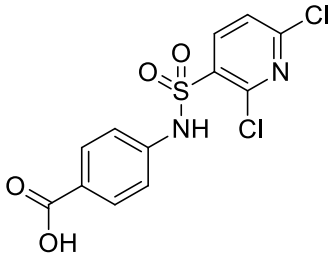


Figure 14. *Results of consensus docking.* Black line, average RSMD of the consensus docking; red line, percentage of survived compounds; blue interrupted line, best average RMSD obtained with single docking using AutoDock

Consensus docking was then used in virtual screening for new PIN1 inhibitors. The 10 docking procedures were applied to a filtered Enamine database, and 32 compounds reached a consensus of 10. These 32 compounds were subjected to molecular dynamic simulations, to examine the stability of their binding. A total of 10 compounds had an average RMSD (between the position of the 32 ligands during the simulation and their initial docking poses) <2.0 Å.

The ten compounds were tested in biological assays to evaluate their PIN1 inhibitory activity. A fluorescent assay with a logarithmic dilution from 1 mM to 1 nM of each compound was used. ATRA

was tested as positive control. The half-maximal inhibitory concentration (IC_{50}) was >100 µM for nine compounds (**Table 1**). One compound (called VS10) had a mean IC_{50} = 13.4 µM (SD = 1.24 µM). The positive control, ATRA showed an IC_{50} of 33.2 µM (in the range of published results (Liao et al., 2017)). Therefore, our compound VS10 had an IC_{50} of about 2-fold less than that of ATRA.

Name	**Structure**	**IC_{50} (µM)**
VS1 27089045		>100
VS2 155306018		>100
VS3 154402827		>100
VS4 376048885		>100
VS5 321764223		>100
VS6 30432390		>100
VS7 311721389		>100

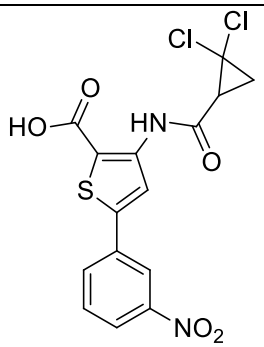
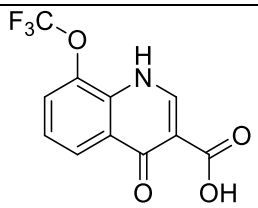
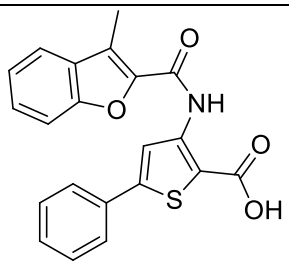
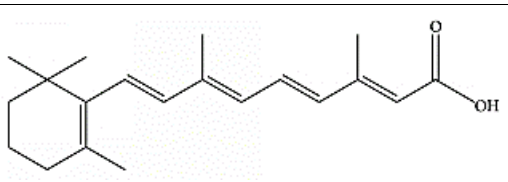
VS8 101198538		>100
VS9 40568444		>100
VS10 101214771		13.4 (1.2)
ATRA		33.2 (1.8)

Table 1. *Molecular IDs, structures and half-maximal inhibition concentration (IC_{50}) on human PIN1 isomerization.* SensoLyte Green assay was performed for 10 compounds selected by virtual screening and for ATRA (positive control). Values are mean (SD).

Figure 15 shows the binding of VS10 in the PIN1 binding site. The carboxylic group of the ligand has ionic interactions with R69 and K63, the thiophene ring interacts with C113, and the phenyl ring is inserted into a lipophilic cleft mainly delimited by L122, M130, F134 and H157. The 3-methylbenzofuran-2-carboxamide fragment makes an H-bond with the hydroxyl oxygen of S154 and is partially exposed to water.

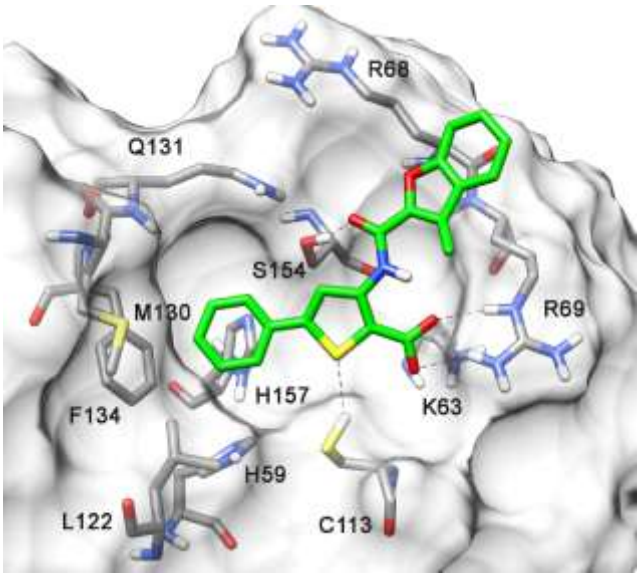


Figure 15. *Putative binding pose of VS10 in the binding site of PIN1*. VS10 is in green.The most relevant ligand-protein interactions are marked.

3.2.2. VS10 inhibits PIN1 in cancer cells

The activity of VS10 was tested in human OVCAR3 and OVCAR5 cell lines, as models of HGS-EOC, and in human SKOV3 and A2780 OC cell lines. Cells were treated with serial dilutions of the drug for 96 h. The IC_{50} values were calculated referring to cell viability. VS10 showed IC_{50} values ranging from 53.9 to 76.4 μM (**Table 2**).

Cell line	IC_{50} (μM)
OVCAR3	53.9 (26.0)
OVCAR5	75.0 (25.7)
SKOV3	76.4 (14.5)
A2780	53.9 (21.5)

Table 2. *IC_{50} of VS10 in ovarian cancer cell lines*. Values are mean (SD).

3.2.3. VS10 down-regulates PIN1 downstream targets

To test the PIN1 specificity of VS10, we tested the effects of PIN1 inhibition on three PIN1 targets in the OVCAR3 cell line. We found that VS10 treatment decreased the levels of β-catenin, cyclin D1 and pS473-Akt proteins in cancer cells (**Figure 16**).

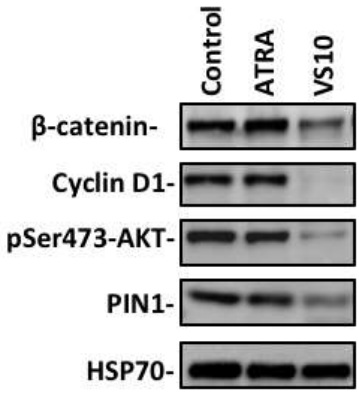


Figure 16. *Compound VS10 has inhibitory effects on PIN1 target proteins.* OVCAR3 cells were treated with 10 μM ATRA (control) or 70 μM VS10 for 24 h, then lysed and subjected to Western blotting. HSP70 was used as a control for sample loading. The image is representative of a single experiment.

In conclusion, VS10 has the potential to be a more efficient PIN1 inhibitor than existing molecules, with possible clinical application in PIN1-overexpressing cancers such as HGS-EOC. Further studies are required to test the efficacy and safety of this molecule.

4. Conclusions and future perspectives

PIN1 was found overexpressed in several cancer types and was approved as biomarker in prostate cancer (Bao et al., 2004) (Ayala et al., 2003). It is an attractive target for cancer therapy, but its function in OC is still unknown.

We showed that PIN1 is overexpressed in human HGS-EOC tissues compared to the normal ones and that PIN1 inhibition affects cancer cell viability. Moreover, PIN1 down-regulation induced cancer cell death via activation of the apoptotic program.
Therefore, our results suggest that PIN1 might be a candidate druggable oncogene in OC.

The neoplastic disease is a multistep process by which normal cells evolve progressively to a malignant phenotype by activation and/or inactivation of several biological mechanisms at the same time, that enable tumor growth and metastatic dissemination (Hanahan, 2014). For this reason, silencing a single oncogene not always gives clinical benefits, but it is necessary to target multiple pathways. Several studies report the effects of PIN1 on the control of key protein expression in the main cancer driving cellular pathways. Understanding the role of PIN1 in OC pathways might permit to plan a multiple inhibition strategy and open the way to new preclinical therapeutic treatment for EOC. Therefore, further studies are required to define the biological network in which PIN1 is involved during OC initiation and progression.

In the last years, there were improvements in OC therapeutic strategies, but the overall survival did not increase significantly because almost all patients develop drug resistance.
Moreover, until now, all the developed PIN1 inhibitors presented some limitations such as poor permeability to cancer cell membrane, low selectivity or efficacy. We identified by consensus docking and molecular dynamic simulations procedures a potential PIN1 chemical inhibitor, the VS10 compound that might represent a new chance for OC patients.

Structurally, VS10 includes a 3-methylbenzofuran-2-carboxamide fragment, a tiophene and a phenyl ring. Each one of VS10 portions interacts with specific regions of PIN1 catalytic site inhibiting PIN1 protein activity at low micromolar range (13.4 ± 1.2 μM).

However, it is known that positive results in biochemical assays do not mean that a compound is efficient in inhibiting the substrate activity in cells due to multiple factors. Interestingly, our

compound presented inhibitory effects on four OC cell lines, with IC_{50} values ranging from 53.9 to 76.4 μM. VS10 showed more inhibitory activity than ATRA, a PIN1 inhibitor recently approved for the treatment of APL and triple-negative breat cancer (Wei et al., 2015).

We established that the VS10 is specific for PIN1 as demonstrated by the decreased levels of PIN1 downstream targets β-catenin, cyclin D1 and pS473-Akt in VS10-treated OC cells. Several studies reported that PIN1 maintains the survival and proliferation of cancer cells throught the regulation of cyclin D1 expression both directly and indirectly as a consequence of its interactions with β-catenin and pS473-Akt (Liao et al., 2009) (Liou et al., 2002) (Ryo et al., 2001). Therefore, the simultaneous alteration of different pathways regulated by PIN1 and involved in cancer progression suggested that VS10 might be a more efficient PIN1 inhibitor than existing molecules.
Further studies are required to test the efficacy, the pharmacokinetic profile and safety of this molecule, but we expect that it might be a new effective targeted drug and support to conventional therapy for OC and PIN1-overexpressing cancer patients.

5. Materials and Methods

5.1. Immunohistochemical analysis

Human ovarian carcinoma and normal ovarian tissue microarrays (OV2001 and OV802 from US Biomax Inc.(Rockville, MD, US)) were incubated with anti-PIN1 antibody (sc-15340) from Santa Cruz (Santa Cruz, CA, US) 1:50, for 1 h at room temperature utilizing the ultraview DAB detection kit with CC1 buffer for 36 min in Benchmark ultra instrument from Ventana Medical Systems (Tucson, AZ, US). The ovarian tissues were analyzed with light microscopy using 10 and 20× magnifications. The immunohistochemical (IHC) staining was converted to an H score: intensity (0, 1, 2, 3) × area (0–100%). The H score from 0 to 75 (first quartile) was defined as low expression and >75 was defined as medium-high expression. Two pathologists scored IHC staining independently.

5.2. Cell culture and lentiviral production

OVCAR3 and SKOV3 human ovarian cancer cell lines from ATCC (Manassas, VA, US). Gustavo Baldassarre (Aviano, Italy) generously provided KURAMOCHI, COV318 and OVCAR5 human ovarian cancer cell lines. Donatella Aldinucci (Aviano, Italy) provided A2780 human ovarian cancer cell line. OVCAR3, SKOV3, KURAMOCHI, OVCAR5 and A2780 cell lines were grown in RPMI-1640 medium with 10% fetal bovine serum. COV318 cell line was grown in DMEM medium with 10% fetal bovine serum. All the cell lines tested negative for mycoplasma contamination by PCR analysis and gel electrophoresis.

To generate knockdown cells, lentiviral particles (LV) were produced. Briefly, 1×10^6 293FT cells (Invitrogen, Carlsbad, CA, USA) were transfected with 2 μg of PAX2 packaging plasmid, 0.5 μg of PMD2G envelope plasmid, and 1 μg of pLKO.1 hairpin vector (control, KD1 or KD2) utilizing 4 μl of Fugene HD (Roche, Indianapolis, IN, USA) on 6-well plates. Polyclonal populations of transduced cells were generated by infection with 1 MOI (multiplicity of infectious units) of shRNA lentiviral particles.

Human PIN1 KD1 shRNA (TRCN0000001033) and KD2 shRNA (TRCN0000010577) from Sigma-Aldrich Merck (Germany).

5.3. Cell viability assay

Three days after LV infection, the cells were seeded in 96-well plates at a density of 10^3 cells/well. The viability was evaluated by CellTiter-Glo®luminescent cell viability assay from Promega (Madison,WI, US) as indicated in manifacture's protocol. Luminescence was read at different time points (0,2,4 and 6 days) using F200 Tecan instrument from Tecan (Switzerland). Averages and standard deviations were obtained from triplicates.

5.4. subG1 flow-citometry analysis

Cells were fixed by adding ice-cold 70% ethanol while vortexing. Fixed cells were stored at 4 °C for at least 2 h and then washed once with PBS. Cells were stained with 1 μg/mL propidium iodide (Roche, Switzerland), 500 ng/mL RNase A (Roche, Switzerland) in PBS and incubated at room temperature for 1 h in the dark. Sub-G1 analysis was performed after 5 days using FACscan instrument from Becton-Dickinson (Franklin Lakes, NJ, US). Data were analyzed with ModFit LTV4.0.5 (Win) software.

5.5. Annexin-V flow-citometry analysis

Annexin V analysis was performed 5 days after lentiviral infection using PE-Annexin V Apoptosis Detection Kit from Becton-Dickinson (Franklin Lakes, NJ, US) according to the manufacturer's protocol. Cells were stained with PE Annexin V and 7-AAD and incubated for 30 min at room temperature in the dark. 300 μL of 1× binding buffer were added to each tube. Samples were evaluated within 1 h by FACS Canto II from Becton-Dickinson (Franklin Lakes, NJ, US). Data were analyzed with BD FACS DIVA software.

5.6. Caspase 3/7 assay

1×10^5 cells were lysed 3 days after lentiviral infection in 10 μL of NP-40 lysis buffer (01M Tris-HCl, 0.01M NaCl, 0.003M MgCl2, 0.03M sucrose, and 0.5% NP-40) and incubated with 10 μL of caspase 3/7 reagent (Caspase 3/7 Glo assay kit from Promega (Madison,WI, US)) for 1 h at room temperature. Luminescence was read at F200 Tecan instrument from Tecan (Switzerland).

5.7. Western Blot Analysis

To check PIN1 down-regulation, OVCAR3, KURAMOCHI and COV318 cells were collected 3 days after lentiviral infection.

To test the effects of the PIN1 inhibitor on PIN1 targets, OVCAR3 cells were seeded in 100 X 20 mm tissue culture dishes (5 X 10^5 cells per dish). One day later, cells were treated with 70 μM VS10 or 10 μM ATRA for 24 h. After treatment, cells were collected for western blotting.

Total cell extracts were obtained by treating cells with RIPA buffer (10mM Tris-Cl (pH 8.0), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl) plus protease and phosphatase inhibitors (Complete-EDTA-free from Roche (Switzerland)). The lysates were incubated on ice for 20 min and sonicated for 5 s. After centrifuging at 13.8×g for 20 min at 4 °C, equal amount of protein (50 μg) was separated by TruePage Precast Gels 4–12% SDSPAGE from Sigma-Aldrich Merck (Germany). Proteins were transferred onto nitrocellulose membranes (Amersham TM Protran TM 0.45 μm NC from GE Healthcare Life Science (Pittsburgh, PA, US)). Free protein-binding sites were blocked for 30 min with 5% non-fat dried milk in TBS containing 0.1% Tween 20 (TBS-T). The membranes were incubated with primary antibodies at 4 °C ON,washed three times with TBS-T and incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Bound antibodies were detected using LiteAblot PLUS Enhanced Chemiluminescent Substrate (EuroClone Life Sciences). The results were analyzed with the ChemiDoc imaging system (Bio-Rad). HSP-70 protein level was used as control. HSP-70 levels remain constant compared to the total protein content under the tested conditions, and its molecular weight differs from those of the proteins of interest.

The antibodies used were: mouse anti-HSP70 (1:1000; cat. no. sc24), rabbit anti-PIN1 (1:250; cat. no. sc15340), mouse anti-human cyclin D1 (1:1000; cat. no. 556470) from BD Pharmingen (Franklin Lakes, USA); rabbit anti-β-catenin (1:1000; cat. no. 8480S), rabbit anti-pSer473-Akt 1:1000; cat. no. 4060s) and rabbit anti- β -actin (1:1000; cat. no. 4967S).
Secondary antibodies were mouse anti-rabbit IgG (1:5000; cat. no. 31464) and goat anti-mouse IgG (1:5000; cat. no. 31432) from Thermo Fisher Scientific.

5.8. Molecular Modeling

The 12 available human PIN1–ligand X-ray complexes were retrieved from the Protein Data Bank (Berman et al., 2000). For all complexes, the ligand was extracted from its X-ray structure and

subjected to a conformational search. To test the reliability of consensus docking in predicting the position of the ligand binding site, each ligand was docked in all the PIN1 3D structures using ten docking procedures, namely AutoDock 4.2.3, DOCK 6.7, FRED 3.0, Glide 5.0 (SP and XP), GOLD 5.1 (ASP, ChemScore, GoldScore and PLP), and AutoDock Vina 1.1, as previously described (G. Poli et al., 2018; Tuccinardi et al., 2015). The reliability of these docking procedures was evaluated in cross-docking analyses. For each procedure, we calculated the average root-mean-square deviation (RMSD) between the position of the ligand predicted by the docking and the known, experimental position, for all the ligands docked into all of the binding sites. The procedure with the lowest average RMSD was considered the most reliable.

To study the effects of consensus docking on the docking evaluations, for each ligand docked into each PIN1 binding site, we clustered the results of the ten docking procedures, to search for common binding modes. For this purpose, consensus level was defined as the number of docking poses that clustered together. At each consensus level, we calculated average RMSD and the percentage of compounds retained ("survived").

To screen for new PIN1 inhibitors using consensus, a hierarchical workflow was used to apply the ten docking procedures to a subset of the Enamine database (HTS Collection) comprising the approximately 32,500 compounds with at least one negative charge. Compounds with a consensus level of ten were selected. To verify the stability of their binding mode as predicted by docking calculations, we did 10 ns molecular dynamic simulations with explicit water. We calculated the average RMSD of the position of each ligand during the simulation compared to their initial docking pose, and analyzed the stability of the interactions predicted by docking. Compounds with an average RMSD <2.0 Å were selected and purchased from Enamine (Monmouth Junction, USA) for study in cellular assays.

5.9. PIN1 inhibitory activity assay

Compounds identified by virtual screening were tested for PIN1 inhibitory activity using the *in vitro* fluorescent SensoLyte Green PIN1 Assay Kit (AS-72240; AnaSpec, Fremont, USA) as indicated in manifacture's protocol. Compounds were serially diluted 1:10 starting from 1 mM to 1 nM. ATRA was used as positive control.

5.10. Half-maximal inhibitory concentration (IC_{50})

The cells were plated in 96-well plates at 5 x 10^2 cells/well. One day later, the cells were treated with VS10 in 1:2 serial dilutions from 300 µM to 2.3 µM. After 96 h, cell viability was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay from Promega (Madison,WI, US)) with the Infinite M1000 PRO microplate reader from Tecan (Switzerland). IC_{50} was calculated using Prism software (GraphPad, USA).

5.11. Statistical analysis

The statistical significance was determined using the two-tails paired t-test, unless specified. A p-value < 0.05 was considered significant for all comparisons done.

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