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Retinoic acid and α-Interferon combination as therapy for Akt-driven non-Hodgkin lymphomas

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A mia nonna Irene,
esempio di forza, tenacia e dedizione al lavoro,
anche se il dolore rimane il tuo dolce ricordo è molto più forte.

To my grandmother Irene,
example of strength, perseverance and dedication to work,
even if the pain remains your sweet memory is much stronger.

The research activity described in this PhD tesis was performed
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ABSTRACT

Given the critical role of the PI3K/Akt pathway in cell growth and survival, it is not surprising that constitutive activation of this pathway contributes to the pathogenesis of many types of lymphoid malignancies, including mantle cell lymphoma (MCL), follicular lymphoma (FL), and cutaneous T-cell lymphoma (CTCL). Available drugs aimed to strike the PI3K/Akt pathway in these tumors are unfortunately dampened by relevant toxicities. Therefore, more effective and safer therapeutic options targeting Akt are needed. Herein we demonstrated that the combination of 9-*cis*-retinoic acid (RA) and Interferon-alpha (IFN-α) induces marked anti-proliferative and pro-apoptotic effects in MCL cells through the modulation of critical targets. Particularly, IFN-α enhances 9-*cis*-RA-mediated G_0/G_1 cell accumulation by down-regulating cyclin D1 and increasing $p27^{Kip1}$ and $p21^{WAF1/Cip1}$ protein levels. Furthermore, 9-*cis*-RA/IFN-α combination induces MCL apoptosis by triggering both caspase 8 and caspase 9 resulting in Bax and Bak activation, and up-regulating the pro-apoptotic proteins Noxa and PLSCR1. In particular, sequestration of the anti-apoptotic proteins Mcl-1 and A1/Bfl1 by up-regulated Noxa results in the activation of Bid, and the consequent induction of apoptosis is inhibited by Noxa silencing. PLSCR1silencing demonstrated a role for this protein not only in 9-*cis*-RA/IFN-α-induced apoptosis but also in 9-*cis*-RA/IFN-α-dependent sensitization to anti-tumor agents currently used in the clinical practice for MCL management, such as doxorubicin and bortezomib. These drugs are able to further increase PLSCR1 expression in 9-*cis*-RA/IFN-α pre-treated MCL cells. Moreover, immunohistochemical analysis of MCL tumor biopsies and primary cultures revealed a variable expression of endogenous PLSCR1 in this setting, an heterogeneity that stimulates the search of possible correlations with clinical-pathological parameters, particularly with those related to the response to pro-apoptotic drugs. In future perspective, in fact, analysis of PLSCR1 expression might allow the identification of tumors more prone to undergo apoptosis, and strategies able to up-regulate PLSCR1, like 9-*cis*-RA/IFN-α combination, might successfully complement and improve conventional treatment modalities. Notably, we also found that 9-*cis*-RA/IFN-α-induced PLSCR1 up-regulation occurs through STAT1 activation in dependence on Akt pathway. In addition, we demonstrated that 9-*cis*-RA/IFN-α co-treatment is able to trigger apoptotic effects also in FL and CTCL cells, and, more interesting, the 9-*cis*-RA/IFN-α-dependent apoptosis is associated with the inhibition of Akt constitutive activation in the different NHL histotypes analyzed. In particular, Noxa up-regulation in MCL, FL, and CTCL lymphoma cells is associated with nuclear translocation of the FOXO3a transcription factor as a consequence of the 9-*cis*-RA/IFN-α-induced Akt but not of mTOR inhibition. Indeed,

pharmacological suppression of Akt, but not of TORC1, induces apoptosis in FL cell lines, and increases Noxa protein levels in MCL cells, supporting the conclusion that inhibition of the Akt pathway, the resulting FOXO3a activation, and Noxa up-regulation are the critical molecular mechanisms underlying 9-*cis*-RA/IFN-α-induced cell death in different type of lymphoid malignancies. These results strengthen the role of Akt as a clinically relevant molecular target and support the potential therapeutic value of RA/IFN-α combination to improve the management of Akt-driven non-Hodgking lymphomas.

INTRODUCTION

1. Non-Hodgkin lymphoma: an overview.

Lymphoid tissues are characterized by a unique level of biological complexity due to the anatomical organization of functionally distinct cell subpopulations and complex processes of genetic modifications required to generate adaptive immune responses. Not surprisingly, this physiological diversity and complexity are mirrored by the broad spectrum of malignancies derived from lymphocytes. Classically, lymphoid tissues can be divided into two types: the central or primary tissues (bone marrow and thymus), in which lymphoid precursor cells mature to a stage at which they can express antigen receptors, and the peripheral or secondary lymphoid tissues (blood, lymph nodes, spleen, and mucosa-associated lymphoid tissues), in which antigen-specific responses occur. These structures enable the development of the immunoglobulin receptor-expressing B-cell lineage, including naive, germinal center, memory B cells, and plasma cells, as well as the T cell receptor-expressing T-cell lineage, including helper, cytotoxic, and regulatory T-cell subpopulations. This spectrum of cell types provides a simplified conceptual framework for understanding the even more complex landscape of lymphoid malignancies, which includes more than 40 distinct tumor types [Dalla Favera, 2012]. Hodgkin lymphoma accounts for about 10% of all lymphomas, and the remaining 90% are referred to as non-Hodgkin lymphomas (NHLs), among which 85-90% arise from B lymphocytes, whereas the reminder derive from T lymphocytes or Natural Killer (NK) cells [Shankland *et al*., 2012]. The different stages that a naive B-cell must pass through in order to become a mature B-cell in the lymph node reflect the variability of B-cell lymphomas and leukemias that may arise from this organ, and may account for the numerical difference compared to T-cell lymphoma subtypes (Figure 1). NHL is the fifth most frequently diagnosed cancer in Europe, with roughly equal numbers of cases in men and women, with a mean age that, for two-thirds of patients, is higher than 60 years [Shankland *et al*., 2012]. In 2009, 10825 people were diagnosed with NHL in Italy, and 4675 patients died of the disease in 2010 [Shankland *et al*., 2012]. Notably, the incidence of the disease has increased dramatically over the last couple of decades, and little is currently known about the causes responsible for this increase. The frequency of specific subtypes of lymphoma varies substantially in different geographic areas. For example, adult T-cell lymphoma is more frequent in east Asia than in other regions, as is nasal NK-cell or T-cell lymphoma associated with Epstein-Barr virus infection, whereas mantle cell lymphoma and follicular lymphoma are more frequent in western Europe and North America. Diffuse large B-

cell lymphoma, by contrast, is common worldwide [Anderson *et al*., 1998]. The most well established risk factor for the development of NHL is immunosuppression. In fact, HIV-infected patients have an increased risk of developing high-grade NHL, often associated with infection by oncogenic herpesviruses. Others patients at increased risk include organ-transplant recipients, patients receiving high-dose chemotherapy with hematopoietic stem-cell transplantation, and those with inherited immunodeficiency syndromes or autoimmune diseases [Shankland *et al*., 2012]. Also infectious agents have a role in development of some lymphomas, either by inhibition of immune function, or by other mechanisms, such as induction of chronic inflammatory response. Although lymphomas usually develop in lymph nodes, they can also present or involve other organs, in which case they are referred to as extranodal lymphomas [Ambinder *et al*., 2010]. In mantle cell lymphoma, for example, spreading of transformed B lymphocytes often involves the gastrointestinal tract, leading to occasional lymphomatous polyposis [Moynihan *et al*., 1996]. NHLs have a wide range of histological appearances and clinical features, ranging from the more indolent follicular lymphoma to the more aggressive diffuse large B-cell and Burkitt's lymphomas. Based on a clinical/pathological point of view, they are classified as low grade (slow growing) or high grade (fast growing). Mantle cell lymphoma is an example of a high-grade lymphoma, while follicular lymphoma and cutaneous T-cell lymphoma are low-grade lymphomas [Shankland *et al*., 2012]. Low-grade NHLs normally progress slowly and it may take many years before the disease progresses or even requires any treatment. However, when treatment is needed, these forms usually cannot be cured by chemotherapy alone. On the contrary, chemotherapy can efficiently control and cure different types of high-grade NHL. Nevertheless, lymphomas unresponsive to chemotherapy are poorly controled and may be fatal. Hence, there is a pressing need to define the molecular mechanisms underlying NHL pathogenesis in order to design therapeutic strategies able to improve the management of these tumors and, therefore, patients outcome.

New molecular parameters are continuously added from clinical and laboratory research to provide useful histopathological classification in order to assist tumor diagnosis and control. For instance, the t(14;18) chromosomal translocation, which causes the juxtaposition of the Bcl-2 gene on chromosome 18 to the transcriptionally active immunoglobulin heavy-chain region on chromosome 14, was identify as the genetic hallmark of follicular lymphoma. This translocation can be detected, in fact, in 80–90% of follicular lymphomas and up-regulates Bcl-2, which increases the apoptotic threshold and prevents programmed cell death [Lenz *et al*., 2010]. In mantle cell lymphoma, the cyclin D1 region on chromosome 11 translocates to the same immunoglobulin heavy locus on chromosome 14, with the consequent cyclin D1 over-expression

and cell cycle alteration [Shankland *et al*., 2012]. Chromosomal translocations are frequently involved in NHL pathogenesis. These genetic alterations are probably the results of abnormal gene recombination events occurring during physiological B-cell development and differentiation, such as somatic hypermutation and class-switch recombination [Lenz *et al*., 2010]. In the case of lymphomas, these genetic events typically results in the presence in the proximity of the chromosomal recombination site of a proto-oncogene, which thus is transcriptionally detregulated . The developmental biology of T-cell lymphomas is less well understood, and most subtypes are not associated with distinct genetic or biological changes. Moreover, the single step of T-cell receptor rearrangement, in contrast to the different steps of programmed genetic modification required for B-cell maturation, accounts for less chances for T-cells to acquire oncogenic alterations than for B-cells. Indeed, recurring translocations that activate specific oncogenes are unusual in T-cell lymphomas, apart from the t(2;5) involving ALK (the anaplastic lymphoma receptor tyrosine kinase gene) seen in anaplastic large cell lymphoma [Shankland *et al*., 2012]. However, detailed studies carried out on different subtypes of lymphoma have shown as these major alterations are not sufficient to explain the complete cell transformation and the aggressive behavior of these tumors. In fact, although the function of the primary protein deregulated by a translocation is an important determinant of the biological behavior of the resulting lymphoma, secondary alterations can modulate this behavior leading to a more aggressive phenotype. Two main groups of oncogenic alterations may be discerned in lymphoma: inactivation of apoptosis and activation of survival pathways, which allow the malignant cells to escape the programmed cell death, and activation of cell proliferation and cell cycle deregulation.

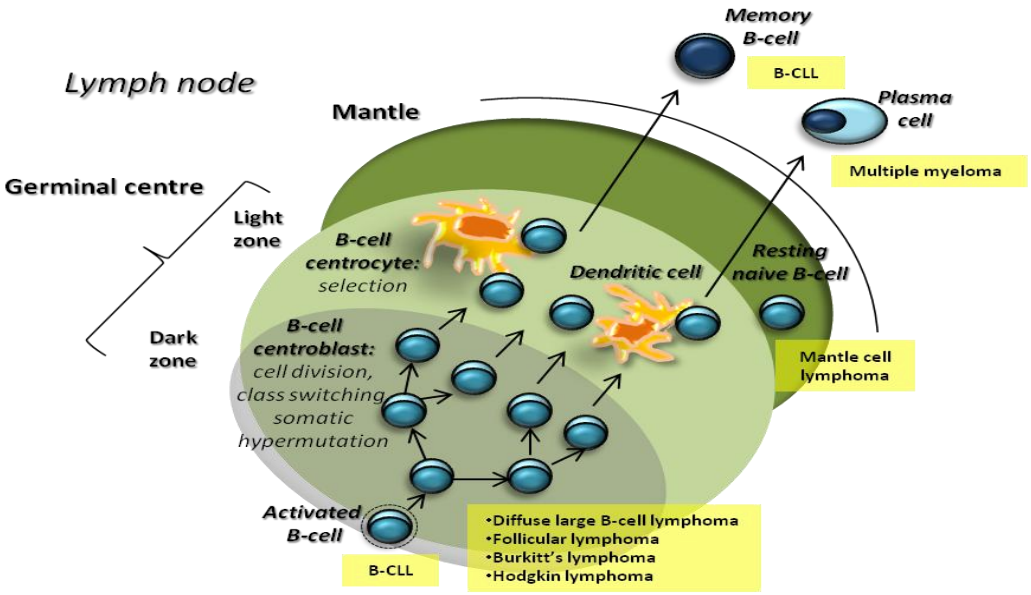


Figure 1. Cellular origins of representative B-cell NHLs.

2. The PI3-K/Akt/mTOR signaling pathway and its role in lymphomagenesis.

One of the major signaling pathway involved in normal cell biology is the PI3-K/Akt/mTOR kinases pathway [Bunney *et al*., 2010]. In physiological conditions, its activation regulates numerous cellular processes, including protein translation, cell size, differentiation, cell cycle progression, motility, apoptosis, angiogenesis, autophagy, and senescence. PI3-K proteins are activated downstream of growth factor receptor signaling and in response to certain metabolic cues. Activated PI3-K phosphorylates the head group of phosphatidylinositol, generating the membrane lipid phosphatidylinositol-trisphosphate (PIP3), which behaves as a secondary messenger favoring the recruitment of the Akt protein to the plasma membrane where this serine/threonine kinase is activated upon phosphorylation. Activated Akt then phosphorylates numerous downstream substrates promoting cell growth, proliferation, and resistance to apoptosis. An immediate consequence of Akt activity is stimulation of mTOR, which is engaged for serine/threonine phosphorylations as part of two distinct complexes, mTORC1 and mTORC2 (Figure 3). When activated by Akt, mTOR promotes cell growth and proliferation by stimulating protein synthesis [Bunney *et al*., 2010]. In particular, it exert its functions by phosphorylating the p70 S6 kinase (S6K), which in turn phosphorylates the S6 ribosomal protein (S6rp), as well as the crucial repressor of the eukaryotic initiation factor 4E (eIF4E), the eIF4E-binding protein-1 (4E-BP1), thus triggering protein translation [Hay *et al*., 2004]. In the un-phosphorylated state, in fact, 4E-BP1 suppresses eIF4E, which is the rate-limiting step in the formation of the large translation initiation complex eIF4F. This complex is essential for the translation of mRNA of many genes, including a variety of potent oncogenes, such as c-MYC and cyclin D1, and genes promoting cell survival, like Bcl-2 and Mcl-1. (Figure 3) [Schatz, 2011]. In addition to receiving signals from Akt, mTOR monitors the cell environment for the presence of growth factors and nutrients. If the cell needs additional nutrients, mTOR can increase their uptake and promote angiogenesis (Figure 2) [Bunney *et al*., 2010]. Because Akt and its signaling partners are so powerful, the cell has adapted mechanisms to tightly regulate their activity. One important watchdog is the tumor-suppressor PTEN phosphatase (Figure 3), which removes the phosphate groups added to membrane phospholipids by PI3-K, thus preventing activation of Akt and its downstream substrates [Vazquez *et al*., 2000]. Not surprisingly, cancer cells frequently exploit the pro-growth and survival end points of PI3-K/AKT/mTOR signaling to their advantage. Indeed, this is one of the most frequently deregulated pathways in human cancer overall, including lymphoid malignancies [Vivanco *et al*., 2002; Samuels *et al*., 2004]. Typical deregulation include mutation or amplification of the PI3-K gene, over-expression and aberrant activation of Akt, loss of function of PTEN or of other tumor-suppressor intermediates.

Increased activity of some growth factor receptors can also enhance the activity of the pathway [Mills *et al*., 2001].

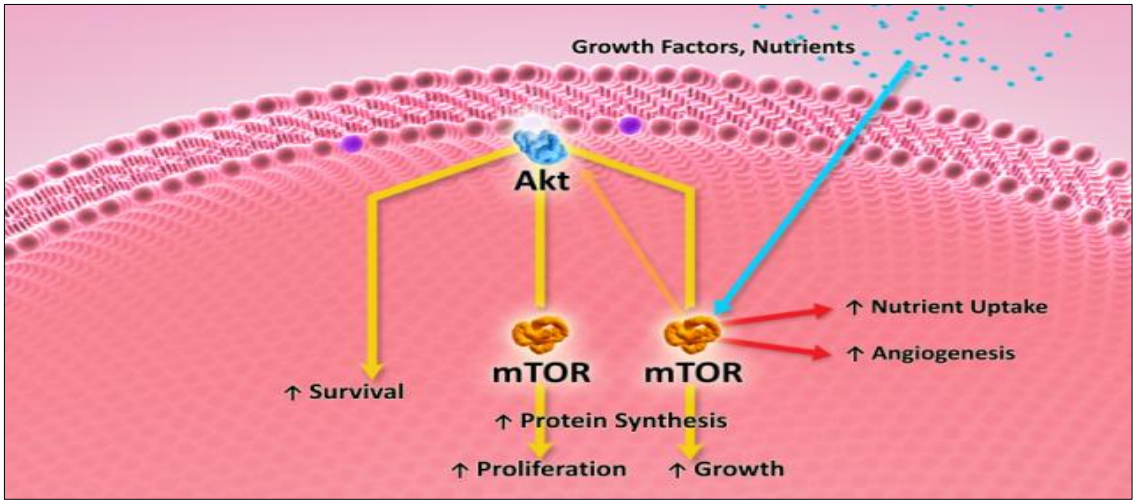


Figure 2. Cross-section of the cell, including part of the membrane and cytoplasm. The protein Akt is associated with a phospholipid in the cell membrane. Arrows represent signaling pathways extended from Akt. The mTOR protein is shown as a part of the "increased proliferation" and "increased growth" pathways. Small blue molecules outside the cell represent growth factor and nutrients, and a blue arrow leads into the cell from these molecules to mTOR, indicating that mTOR protein monitors the levels of these molecules.

Multiple drugs targeting the PI3-K/AKT/mTOR pathway are now either approved or under development as cancer therapies. Of these, the most thoroughly studied in clinical trials are the rapamycin-analogs (rapalogs) mTOR inhibitors that target mTORC1, of which temsirolimus and everolimus are now approved for advanced renal cell carcinoma [Fasolo *et al*., 2011]. The prototype, rapamycin (sirolimus), enters the cell and binds to a protein called FKBP12. This complex, in turn, binds to and inhibits mTOR. Inhibition of mTOR with rapamycin has been found to decrease the oncogenic transformation of human cells induced by PI3-K or Akt, and has *in vivo* anti-tumor effects in murine lymphoma models with Akt activation [Bjornsti *et al*., 2004]. NHL is one of the major tumor types where the rapalogs have been extensively assessed and show significant clinical activity. For example, three rapamycin derivatives, temsirolimus, everolimus, and deforolimus, are being tested in clinical trials in patients suffering from different B-cell NHL histotypes, showing improved bioavailability but, in contrast to rapamycin, less immunosuppression [Tay *et al*., 2010]. Larger trials reported over the last 2 years highlighted both the potential of targeting PI3K/Akt/mTOR signaling in NHLs and the many challenges that must be overcome to make the rapalogs work better and in a larger numbers of patients.

2.1 Why targeting PI3-K/Akt/mTOR pathway in NHL: the case of mantle cell lymphoma, follicular lymphoma and cutaneous T-cell lymphoma.

PI3-K/Akt/mTOR activation occurs in a substantial proportion of NHL histotypes [Drakos *et al*., 2008], including mantle cell lymphoma (MCL), follicular lymphoma (FL), and cutaneous T-cell lymphoma (CTCL) [Dal Col *et al*., 2008; Marzec *et al*., 2008; Bhende *et al*., 2010].

Gene expression profiling and proteomic studies have demonstrated that MCL cells carry a profound deregulation of several members of the PI3-K/Akt pathway, and evidence has also been provided suggesting that Akt- and mTOR-dependent signaling is constitutively activated in this lymphoma [Martinez *et al*., 2003; Dal Col *et al*., 2008]. Notably, activation of Akt and mTOR was detected both in cell lines and tumor biopsies derived from patients with MCL, demonstrating that these kinases are inherently activated also *in vivo* [Dal Col *et al*., 2008]. Such activation seems to be associated with abnormal levels of expression of the phosphorylated/inactivated form of the phosphatase PTEN [Dal Col *et al*., 2008] or with a loss of its expression [Rudelius *et al*., 2006]. These alterations, together with the high level of genomic instability that characterizes MCL, may account for the aggressive clinical behavior of this lymphoma and for its poor response and resistance to conventional chemotherapy. MCL represents 5-10% of all NHLs and is characterized by advanced stage at presentation and frequent extranodal localizations. Patients' median age at diagnosis is 58 years and the median overall survival is 3-4 years [Campo *et al*., 1999]. Although a few patients are asymptomatic and can be observed for a period without treatment, most patients are symptomatic and need treatment at diagnosis. In any case, MCL is among the lymphomas with poorest prognoses [Zain *et al*., 2010]. Over-expression of cyclin D1, as a consequence of the t(11;14)(q13;q32) translocation, is the hallmark of MCL, being detected in >95% of cases. Cyclin D1 and subsequent cell cycle deregulation, however, are not sufficient for lymphomagenesis [Klier *et al*., 2008], but cooperation with pathogenic microenvironmental stimuli, such as IL-4, IL-10, and CD40 activation [Visser *et al*., 2000], as well as additional genetic changes, are required to induce and sustain the transformed phenotype of mantle cells. In fact, defects involving inhibitors of G1-S cell cycle progression, such as p53, $p27^{Kip1}$, $p16^{INK4a}$, $p15^{INK4}$, and $p21^{WAF1/Cip1}$ may also occur in MCL [Jadayel *et al*., 1997]. Notably, the PI3-K/Akt pathway controls the expression of cell cycle regulatory proteins, such as $p27^{Kip1}$ and cyclin D1. In particular, threonine 157 phosphorylation of $p27^{Kip1}$ by Akt delocalizes the protein in the cytoplasm, thus preventing its inhibitory functions and favoring its proteasome-mediated degradation [Viglietto *et al*., 2002]. Indeed, $p27^{Kip1}$ phosphorylated on threonine 157 was detected in MCL cells showing constitutive Akt activation [Rudelius *et al*., 2006]. It is also

known that Akt can control $p27^{Kip1}$ expression through the negative regulation that exerts on the members of the Forkhead family of transcription factors (FOXO) [Tizivion *et al*., 2011]. These proteins, in fact, are responsible for the direct transcriptional activation of the CDKN1B gene, which encodes for the cell cycle inhibitor $p27^{Kip1}$ [Medema *et al*., 2000]. FOXOs transcriptional activity is regulated by the control of their intracellular localization and, in particular, they become inhibited upon Akt-dependent phosphorylation as this status causes their retention into the cytoplasm abolishing nuclear translocation [Tizivion *et al*., 2011]. However, in MCL cells, the amount of $p27^{Kip1}$ protein seems to be regulated mainly at post-transcriptional level by the ubiquitin-proteasome machinery [Guidoboni *et al*., 2005; Dal Col *et al*., 2008]. In addition, Akt can control the levels of cyclin D1 through the inhibithory phosphorylation of GSK-3β, a kinase that negatively regulates cyclin D1 expression at both the transcriptional and post-transcriptional levels [Diehl *et al*., 1998]. Because of the involvement of mTOR in cyclin D1 translation [Schatz, 2011], it was postulated that MCL cells might show particular susceptibility to mTOR inhibitors. In line with this, preclinical studies showed that mTOR inhibition results in decreased cyclin D1 expression and, subsequently, in cell proliferation blockade [Peponi *et al*., 2006; Dal Col *et al*., 2008].

In FL, two studies reported that Akt phosphorylation at serine 473 was significantly more common in FL tumor cells than in non-malignant B cells derived from follicular hyperplasia [Zha *et al*., 2004; Gulmann *et al*., 2005]. FL is the second most common lymphoma in the USA and western Europe, accounting for about 20% of all NHL. The median age at diagnosis is 60 years [Glass *et al*., 1997]. This lymphoma often presents with painless peripheral lymphadenopathy, which may increase and decrease in size. Staging investigations usually identify disseminated disease at diagnosis, with involvement of the spleen (in 40% of cases), liver (50%), and bone marrow (60–70%) [Dalla Favera, 2012]. The clinical course can vary: some patients might not need treatment for several years, whereas others, with massive nodal or organ involvement, need intervention. The median survival of patients presenting advanced stage FL is 10 years, although survival seems to have increased since the adoption of therapeutic monoclonal antibodies [Swenson *et al*., 2005]. Although usually indolent, 10–70% of cases may undergo histological transformation to an aggressive DLBCL, and these cases are associated with a poor prognosis [Freedman *et al*., 2010]. Acquisition of additional mutations, such as p53 gene mutation, seems to be linked to this transformation [Bhende *et al*., 2010]. Unfortunately, although FL is sensitive to some chemotherapeutic agents, relapse is common and transformation to DLBCL is considered incurable with conventional chemotherapy. Recent studies showed PI3-K/Akt/mTOR activation downstream of tumor necrosis factor superfamily receptor signaling,

and demonstrated that ligands for these receptors were commonly expressed in tumor microenvironment [Gupta *et al*., 2009]. Activation of mTOR downstream of Syk signaling in FL was seen in another study [Leseux *et al*., 2006]. Considering that Akt can also further up-regulate Bcl-2 expression through cAMP-response element-binding protein [Bhende *et al*., 2010], thus providing a constitutive survival signal for FL, the activated PI3-K/Akt/mTOR pathway may represent a potential therapeutic target for the treatment of this lymphoma. Interestingly, preclinical studies demonstrated how NVP-BEZ235, a dual PI3-K and mTOR inhibitor, is effective in inhibiting the growth of transformed FL cell lines characterized by constitutive activation of both Akt and mTOR [Bhende *et al*., 2010].

Recent investigations showed how PI3-K/Akt-dependent mTOR activation is impaired also in a subtype of T-cell NHL, the CTCL, which comprises a heterogeneous group of lymphoproliferative disorders characterized by clonal expansions of mature, post-thymic $CD4^+$ T-cells that infiltrate the skin [Girardi *et al*., 2004]. The most common histotypes of CTCL are mycosis fungoides and its leukaemic variant, the Sézary's syndrome. Although initially indolent, CTCL shows the tendency to progress to more aggressive forms with high morbidity, limited response to therapy, and poor prognosis [Hwang *et al*., 2008]. CTCL cells display mTORC1 activation, with the highest percentage of positive cells identified at late, clinically aggressive stages of cell transformation. Notably, inhibition of mTORC1 as well as PI3-K/Akt signaling profoundly impairs the proliferative capacity of CTCL-derived short-term cultures and cell lines [Marzec *et al*., 2008]. Taken together, these findings support the "operational" inclusion of MCL, FL, and CTCL among the lymphoid malignancies characterized by inherent PI3-K/Akt/mTOR signaling pathway activation and, consequently, potentially responsive to the treatment with PI3-K/Akt/mTOR inhibitors.

2.2 Current and emerging therapeutic approaches targeting the PI3-K/Akt/mTOR pathway.

As single agents, mTORC1 inhibitors are usually more effective in NHLs than in many other cancers, although they have not been clinically successful as expected from preclinical studies [Wang *et al*., 2009]. Their activity is highly specific for the mTORC1 complex and does not substantially affect the activity of mTORC2.

Based on encouraging effects seen during phase I clinical trials and on the preclinical rationale discussed above, according to which mTOR kinase regulates cyclin D1 expression, the first phase II evaluation of temsirolimus in NHL was carried out in MCL patients [Witzig *et al*., 2005; Ansell *et al*., 2008]. This study and a subsequent multinational phase III randomized study

allowed the identification of an effective and less toxic clinical dose for temsirolimus monotherapy, leading to its approval in 2009 as a therapeutic option for relapsed/refractory MCL [Hess *et al*., 2009]. However, the objective response rate was only 22%, and was associated with a median progression-free survival of 4.8 months. Similarly, everolimus and deforolimus were tested as single agents in a phase II clinical trial involving relapsed/refractory MCL patients, in which these drugs led to an overall response rate of 30%. Comparable activity was observed in FL patients treated with everolimus in a phase II clinical trial, whereas temsirolimus showed more promising results [Smith *et al*., 2010; Witzig *et al*., 2011]. Nevertheless, response rates of rapalogs as single-agents are typically below 50% in NHLs, resulting in remissions that are neither complete nor durable. Furthermore, toxicity of rapalog therapy in NHLs is generally high, though responsive to dose reductions. Hematological toxicities are the most common, especially thrombocytopenia, with other side effects seen at lower rates, including skin rush, fatigue, weight loss, and gastrointestinal complications [Schatz *et al*., 2011]. Another important challenge with the mTORC1 inhibitors is the frequent emergence of resistance, which may be due to the fact that rapamycin/rapalogs mTORC1-dependent inhibition leads to feedback activation of upstream components of the PI3-K/Akt/mTOR pathway by at least two different ways. First, normal activation of the downstream target of mTORC1, S6K, leads to feedback inhibition of upstream PI3-K/Akt activation through repression of the signaling molecule involved in cell growth, IRS1 (Figure 3). Disruption of mTORC1 by rapalogs blocks this feedback mechanism, allowing upstream activation of Akt [O'Reilly *et al*., 2006]. Second, as the rapalogs leave mTORC2 largely unaffected, this second mTOR-kinase complex can phosphorylate Akt at serine 473 (Figure 3), thus compensating for mTORC1 inhibition [Feldman *et al*., 2009]. These dual mechanisms have been studied *in vitro* and, to a lesser extent, in tumor samples. Their actual relevance as rapalogs resistance mechanisms in patients, however, remains to be established. Certainly, clinical experiences with rapalogs in NHL to date highlight the need to identify new biomarkers of response and resistance that may inform the design of alternative curative approaches able to bypass resistance mechanisms. Notably, the newly developed TOR-kinase inhibitors, like the competitive ATP inhibitor Torin 1, specific for shutting down the activity of both mTORC1 and mTORC2 simultaneously, are designed to bypass, at least in part, the 'collateral' rapalogs-dependent Akt activation.

As the treatment with rapamycin-type compounds typically results in a stabilization of the disease or partial remission rather than in tumor elimination, combinations with other agents, both standard cytotoxic drugs or other targeted therapies, that ideally would result in the complete remission and cancer cure, are now intensively investigated. Strong preclinical data

provide support, for example, for rapalog-chemotherapy combinations [Schatz *et al*., 2011]. In addition, combining rapamycin with chemotherapy was shown to completely bypass the ability of Akt to promote chemotherapy resistance in mouse lymphoma models [Wendel *et al*., 2004]. There are now several trials planned or underway for NHL patients based on the combination of chemotherapeutic agents, currently used in the clinical practice, with the rapalogs, including temsirolimus and rituximab or bendamustine for relapsed FL, temsirolimus plus R-cladribine, rituximab, or bortezomib for previously untreated MCL, and everolimus plus CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) for peripheral T-cell lymphoma [Schatz *et al*., 2011]. Though some combinations seem promising based on preclinical studies, most of the attempts to combine mTORC1 inhibitors with other drugs in clinical trials have been rather disappointing, also because of occasional drug antagonism [Marzec *et al*., 2011].

Another approach that exploits the PI3-K/Akt/mTOR pathway is the direct targeting of PI3 or Akt kinases. Multiple new inhibitors with this function are under development, with ongoing phase I trials, also including NHL patients. Many of the PI3-K inhibitors have specificity for particular isoforms of its catalytic subunit, allowing a high degree of selectivity of action to prevent side effects from off-target inhibition. For example, the PI3K inhibitor CAL-101, highly specific for the δ isoform expressed almost exclusively in cells of hematopoietic origin [Lannutti *et al*., 2011], is under evaluation in five different trials with NHL patients, including MCL, with preliminary reports of strong activity [Schatz, 2011]. Moreover, a phase II evaluation in combination with rituximab is ongoing in patients with previously untreated FL [Schatz, 2011]. Among Akt inhibitors, encouraging phase II data have emerged for perifosine in multiple myeloma, with phase III studies of the agent ongoing. It was also demonstrated that MK-2206, an allosteric Akt inhibitor established in phase I studies, decreased the kinase phosphorylation in whole blood, and the agent GSK2141795, which inhibits all Akt isoforms at nanomolar concentrations, is being assessed in a phase I trial enrolling lymphoma patients [Pal *et al*., 2010]. Finally, perifosine have been tested *in vitro* demonstrating pro-apoptotic effects in lymphoma cell lines [Chiarini *et al*., 2008].

A recent study by Dal Col *et al*., conducted on MCL cell lines and tumor biopsies using Akt or mTORC1 inhibitors, demonstrated how constitutive activation of Akt and mTOR in MCL may have distinct functional significance. According to this work, inhibition of Akt or mTORC1, resulting in accumulation of MCL cells in G_0/G_1, not only blocks basal cell proliferation, but also antagonizes the growth-promoting activity exerted by CD40 activation and IL-4 co-stimulation in primary MCL cultures. Regardless of the inhibitor used, these effects are associated with cyclin D1 down-regulation through proteasome-dependent degradation, and with increased

$p27^{kip1}$ expression levels thanks to the enhanced stability of the protein. Nevertheless, Dal Col and coworkers demonstrated that TORC1 inhibition results in cyclin D1 down-regulation only in MCL cells in which the GSK-3β kinase is under the direct control of mTOR. Conversely, MCL cell lines resistant to rapamycin-induced inhibition of GSK-3β phosphorylation do not down-regulate cyclin D1 following TORC1 inhibition [Dal Col *et al*., 2008]. These findings could help reconcile the discrepant results obtained so far with rapamycin in different MCL cell lines, and may account also for the highly variable efficacy that single mTORC1 inhibitors have shown in clinical trials. In keeping with the notion that the PI3-K/Akt pathway critically regulates cell survival, the same investigators found that inhibition of this cascade may induce MCL cell apoptosis, involving both the intrinsic and the extrinsic apoptotic pathways. It is worth mentioning that PI3-K inhibition induces apoptosis in primary MCL cultures as well, although with markedly variable apoptotic responses that confirm the clinical observation of the heterogeneity that characterizes this lymphoma. Interestingly, significant levels of apoptosis have been observed in MCL cultures after PI3-K inhibition also in the presence of costimulatory signals, such as CD40 triggering and IL-4, further strengthening the potential therapeutic effectiveness of targeting PI3-K/Akt. On the other hand, rapamycin induces no or only limited apoptotic responses in either established cell lines or primary MCL cultures [Dal Col *et al*., 2008].

Comparable results were recently obtained by Bhende *et al*. on FL cell lines. They showed that a dual PI3-K/mTOR inhibitor, NVP-BEZ235, is able to block FL cell proliferation through increased apoptotic responses, an effect that was not observed in FL cells treated with rapamycin alone. In particular, reduced phosphorylation of the downstream target of mTORC1, S6K, indicates that NVP-BEZ235 inhibited protein synthesis, whereas reduced phosphorylation of Akt at serine 473 suggests that mTORC2 feedback phosphorylation of Akt was also inhibited. Additionally, they found that NVP-BEZ235 and bortezomib synergize to inhibit FL cell lines proliferation, suggesting that this drug may have significant potential for the treatment of this lymphoma [Bhende *et al*., 2010].

Similarly, a cytostatic rather than a cytotoxic effect of mTORC1 inhibitors was shown by Marzec *et al*. in CTCL-derived short-terms and cell lines. Also in this case, in fact, inhibition of PI3-K/Akt but not of mTORC1 alone significantly affects cell survival. However, simultaneous inhibition of mTORC1 and either PI3-K/Akt or MEK/ERK enhanced CTCL apoptotic cell rates [Marzec *et al*., 2011]. This observation further supports the possible relevance of combinations with other compounds able to induce apoptosis either alone or together with an mTOR inhibitor,. These findings are quite interesting in the light of planning future clinical trials enrolling CTCL

patients for the study of agents targeting PI3-K/Akt and mTOR signaling pathway. Indeed, these drugs are particularly needed if we consider that the response rates to most of the chemotherapeutic agents currently used to treat CTCL, including gemcitabine, doxorubicin, and bortezomib, are only of 25-50%, and that, other than allogeneic stem cell transplant, there are no curative therapies for this disease [Lansigan *et al*., 2010].

The important relationships between PI3-K/Akt/mTOR pathway intermediates described so far, and some of the drugs targeting it, are illustrated in a simplified diagram in Figure 3.

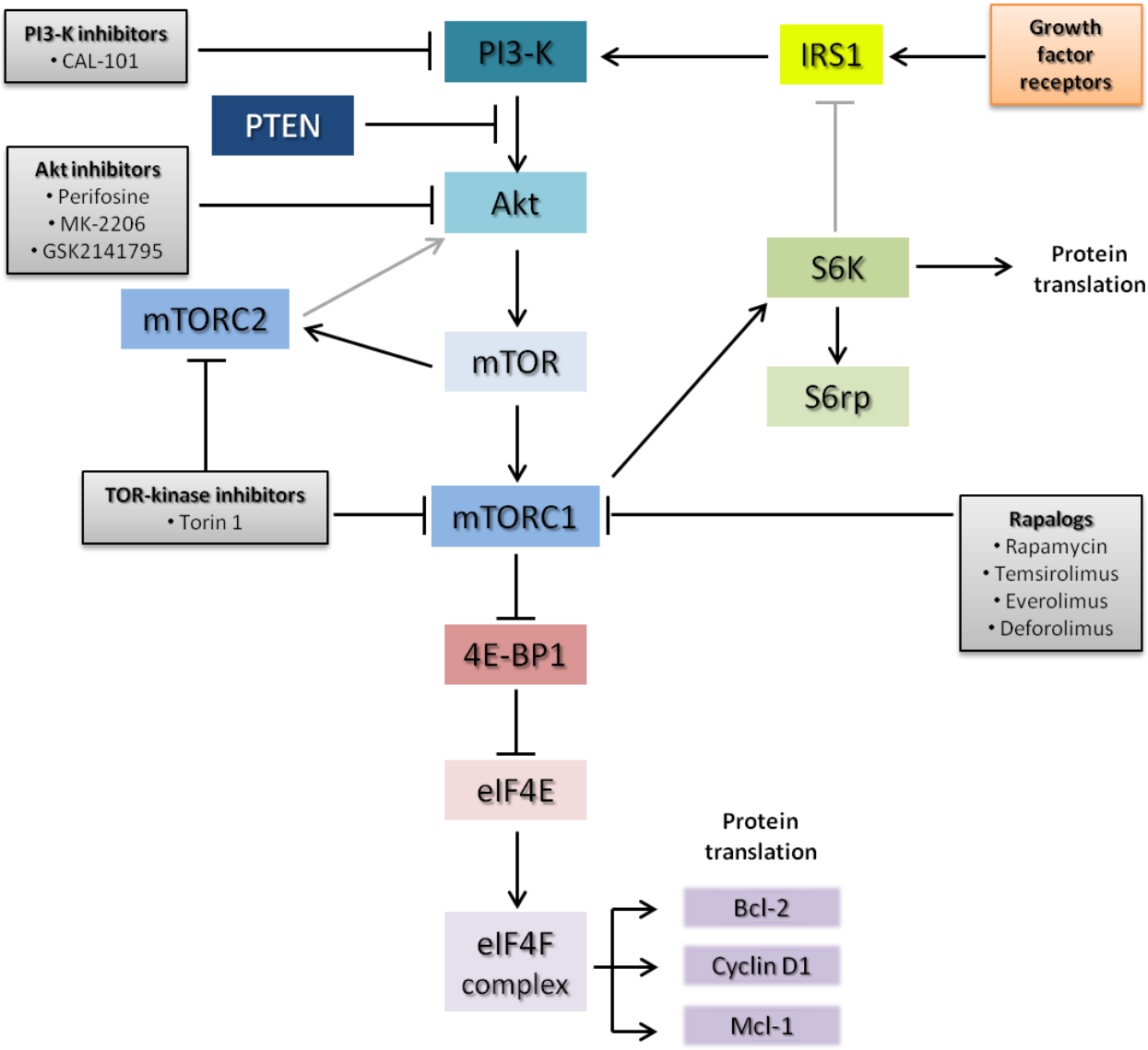


Figure 3. The PI3-K/Akt/mTOR pathway. Diagram schematically illustrates important relationships between pathway intermediates and shows the point at which various drug classes act. Resistance mechanisms to the rapalogs discussed in the text are shown with grey indicators (modified by Schatz, 2011).

Summing up, available evidence suggests that the Akt kinase may constitute a more effective target for those NHLs characterized by inherently PI3-K/Akt/mTOR pathway activation compared to mTORC1, since Akt but not mTOR inhibition triggers extensive apoptotic responses. Nevertheless, considering the large spectrum of basic biological cellular functions regulated by Akt activity, the main problem resulting from the use of its inhibitors in clinical practice remains the exceedingly high load of side effects, although many studies have been designed to increase their efficacy while reducing toxicity. The dose-limiting toxicities for the oral compounds, for example, are malaise and weight loss, consistent with the Akt inhibitors interference with the metabolism of glucose [Luo *et al*., 2005]. However, in perspective, there is a strong rationale to keep believing that targeting Akt will find additional roles in improving outcomes for NHLs patients. The promise of targeting this pathway in NHL, therefore, lies in exploring alternative, carefully selected combinations of new and/or old drugs with more effective but less toxic activities.

3. Retinoids and Interferons: re-evaluating "old" drugs and their combination.

During the past two decades, the anti-proliferative ability of retinoids has been extensively studied, enough to make earn to these compounds a now well-established role in cancer therapy, including lymphoma therapy. In parallel, Interferons have been demonstrated to inhibit tumor cell growth and induce apoptosis, thanks also to their ability to control several pathways such as the Akt pathway. Combinations of these two compounds, with well-known and usually tolerated and manageable adverse effects, have been widely used in clinical practice, and, by virtue of their pleiotropic activities and cellular targets, may constitute an attractive strategy also for the treatment of the Akt-driven NHLs.

3.1 Retinoids and their role in cancer therapy.

Retinoids are a class of compound structurally related to vitamin A (retinol) including both natural and synthetic analogs. They exert profound effects on a wide array of physiologic processes, including embryonal morphogenesis, visual response, regulation of cell proliferation and differentiation, epithelial cell and bone tissue growth, and immune function [Guidoboni *et al*., 2005]. These multiple effects are mediated by the binding to and activation of two different families of specific nuclear receptors: the retinoic acid (RA) receptors (RAR-α, -β, -γ), and the retinoid X receptors (RXR-α, -β, -γ). The activated receptor then activates or represses

transcription of genes containing a retinoic acid response element, responsible for RA biological function. Two or more receptors may cooperate to regulate transcription in the form of heterodimers or homodimers. The variety of receptors, each one having specific transcriptional properties on different promoters, together with the considerable variability of tissue distribution of RARs and RXRs, account for the pleiotropic action of retinoids [Bollag *et al*., 1992].

A correlation between vitamin A and cancer was first noted in the nineteen twenties, when experimentally-induced vitamin A deficiency was shown to lead to hyperplastic, metaplastic, and dysplastic tissue changes, until preneoplastic lesions and, ultimately, neoplasms [Bollag *et al*., 1992]. Forty years later, a preventive effect of vitamin A on the development of chemically induced tumors was demonstrated in animal models [Bollag *et al*., 1992]. Further experiments showed that, in addition to its preventive action, vitamin A and its natural metabolite, the all-trans retinoic acid (ATRA), as well as other synthetic retinoids, also had a therapeutic effect in cancer [Mayer *et al*., 1978], laiding the foundation for the clinical use of retinoids in the therapy of a variety of neoplastic diseases. The undesirable side effects linked to hypervitaminosis A, due to the high doses required to obtained successful responses, led to the development of retinoids with an improved risk/benefit ratio. Nowadays, more than 2500 retinoids have been synthezised and biologically tested. Among the ones of first generation, the ATRA, 13-*cis*-RA and 9-*cis*-RA were identified as promising candidates for clinical trials. 9-*cis*-RA is a pan-RAR and -RXR stereoisomer, whereas the others can bind only RARs [Allenby *et al*., 1993]. It is well established that the anti-tumor activity of retinoids is at least partially due to either induction of cellular differentiation and/or inhibition of cell proliferation [Nickoloff *et al*., 1985]. For example, ATRA and other retinoids have been seen to induce differentiation in acute promyelocytic leukemia, and neuroblastoma human cell lines [Sidell, 1982]. Noteworthy, a recent work carried out on a human neuroblastoma cell line sought out that RA-dependent differentiation confers higher tolerance towards neurotoxins by quickly up-regulating survival signaling, including the Akt pathway [Cheung *et al*., 2009]. Potent anti-proliferative activities of several retinoids were observed in a series of transformed cell lines, including mammary, melanoma, lymphoid and squamous cell carcinoma cell lines [Lotan *et al*., 1980]. Studies comparing RA isomers with distinct ability to bind RARs and RXRs revealed how 9-*cis*-RA was markedly superior to 13-*cis* or ATRA in inducing differentiation, whereas no marked difference was found as concerns the anti-proliferative capacity [Bollag *et al*., 1992]. Several tumor types were used to study retinoids effects as single agents in cancer therapy, including melanomas, squamous carcinomas of the head and neck, and acute promyelocytic leukemia (APL). Moreover, retinoids were shown to have some activity also in hematologic malignancies

different from APL, including juvenile chronic myeloid leukemia, myelodysplastic syndrome, and CTCL [Guidoboni *et al*., 2005]. While results from the treatment with 13-*cis*-RA of melanomas and squamous carcinomas of the head and neck were not successful, those in patients with APL and CTCL were of great interest. APL represented the first clinical model where a high rate of complete remissions has been achieved with single agent retinoid therapy. In particular, ATRA gave spectacular results, with the 95% of APL patients achieving complete remission. The most frequent side effects were those associated with hypervitaminosis A syndrome (dry skin, headache, increase of cholesterol and triglycerides) of usually mild degree, and serious complications encountered by 10-15% of patients after treatment, consisting of hyperleukocytosis, fever, acute respiratory distress, hypotension, renal failure, and thrombosis, were successfully solved with corticosteroids [Huang *et al*., 1988]. In a first study on CTCL, a total of 107 patients in various stages of the disease and some of them pretreated, were treated with 13-*cis*-RA or etretinate. Eighteen complete and 48 partial remission were observed, which compared favorably with conventional therapy [Fitzpatrick *et al*., 1986]. The third generation retinoid bexarotene is currently used in patients who have advanced or refractory CTCL, as it has been shown to be effective and well tolerated, with predictable adverse effects including elevation in serum lipids and cholesterol, and suppression of thyroid function [Lansigan *et al*., 2008, Lansigan *et al*., 2010].

The limited and controversial knowledge regarding the potential efficacy and toxicity of retinoids in the control of B-cell lymphoproliferations stimulated the more in-depth studies carried out in the last years on B-cell malignancies. In this field, Pomponi *et al*. demonstrated how RA may induce marked anti-proliferative responses in EBV-immortalized and fully neoplastic B lymphocytes [Pomponi *et al*., 1996]. Importantly, concentrations corresponding to those achievable in humans following oral administration (1 μM) were able to induce an irreversible inhibition of proliferation. Subsequently, other detailed studies showed that retinoid-induced growth inhibition was the consequence of a direct modulation of the levels of cell cycle regulatory proteins. In particular, RA can drastically reduce the levels of different cyclins while inducing a marked increased stability of the cell cycle inhibitor $p27^{Kip1}$ in lymphoblastoid B-cell lines (LCL) [Zancai *et al*., 1998]. In other cellular systems, the anti-proliferative effect of RA was related to enhanced cyclin D1 ubiquitination and proteolysis, and increased $p21^{WAF1/Cip1}$ transcription [Liu *et al*., 1996, Spinella *et al*., 1999]. More recent studies provided evidence of a pro-apoptotic activity of retinoids, often correlated with the modulation of genes regulating apoptosis, such as Bcl-2 [Pettersson *et al*., 2002]. Interestingly, RA can inhibit cell growth with stimulation of apoptosis also in aggressive lymphomas [Sundaresan *et al*., 1997]. These results

led our group to investigate the effects of retinoids in different types of B-cell NHLs, demonstrating heterogeneous responses in cell lines derived from Burkitt's lymphoma, while interesting findings were obtained in MCL cell lines and primary cultures. A marked anti-proliferative effect, in fact, is induced in MCLs by all three RA isomers, the 9-*cis*-RA, 13-*cis*-RA, and ATRA, although 9-*cis*-RA is the most effective. However, unlike what observed in other cellular systems, this effect is not due to a down-regulation of cyclin D1, but rather to a marked up-regulation of the cell cycle inhibitors $p27^{Kip1}$ and $p21^{WAF1/Cip1}$. Similarly to what was observed in LCLs [Zancai *et al*., 2005], RA-induced $p27^{Kip1}$ up-regulation in MCL cells does not involve transcriptional mechanisms but is the result of an enhanced stability of the protein through the inhibition of ubiquitination and proteasome-dependent degradation. In this respect, RA counteracts the inherently enhanced degradation of $p27^{Kip1}$ that characterizes a significant proportion of typical MCL cases [Guidoboni *et al*., 2005]. Even more relevant in terms of potential clinical application of RA in this setting is the demonstration that RA isomers also inhibit the growth-promoting effect induced by CD40 activation and IL-4 in primary MCL cultures as well as in cell lines [Guidoboni *et al*., 2005]. Given the already established relevance of CD40 activation in the pathogenesis of MCL, indicated also by the presence of CD40-ligand positive cells infiltrating MCL microenvironment *in vivo* [Visser *et al*., 2000], these findings make these compounds highly attractive in terms of potential clinical usefulness. Of note, results concerning the mechanisms underlying RA activity on MCL described so far overlap with those subsequently obtained by our group using specific inhibitors of the PI3-K/Akt/mTOR pathway, though the latter affect also cyclin D1 expression and MCL survival [Dal Col *et al*., 2008]. Indeed, despite the marked anti-proliferative effect, RA-mediated MCL cell growth inhibition is not associated with increased apoptosis [Guidoboni *et al*., 2005]. This consideration make even more interesting the analysis and characterization of the signaling pathways underlying the mechanism of action of RA isomers, to better define their role in cancer therapy, and also to identify possible compounds that, once combined with RA, are able to strengthen its efficacy through the induction of apoptosis. Improved therapeutic results can be expected from the combination of retinoids with other anti-tumor agents generating a synergism between anti-proliferative, differentiation-inducing and apoptotic effects, like cytokines. The combination of different types of retinoids and Interferons, for example, is already widely used in clinical practice, where the two compounds seem to exert synergistic anti-tumor activity in many types of malignancies [Bollag *et al*., 1992; Lansigan *et al*., 2008].

3.2 Activity, clinical application, and targets of Interferons.

Interferons (IFNs) are a family of cytokines with a wide spectrum of biological activities, including anti-viral, immunomodulatory, and growth inhibitory effects [Pestka *et al*., 1987]. They are divided into two major group, the type I IFNs (including IFN-α, β, ω, τ, ζ) and the type II IFN (including IFN-γ), based on the receptor that can bind, and generally have distinct, non-redundant, biological functions. For instance, IFN-γ, mainly produced by activated T-cells and Natural Killer cells, functions primarily as an immune modulator responsible for pathogen clearance, rather than an antiviral agent as, instead, IFN-α and β. Because of their effects, some IFNs subtypes have been extensively used over the years for the treatment of various malignancies, viral infections, and neurologic disorders [Parmar *et al*., 2003]. For example, different types of IFNs are employed in clinical practice for the treatment of patients suffering from chronic hepatitis B and C and osteoporosis, as well as in the control of multiple sclerosis and autoimmune disorders [Baron *et al*., 1991; Weinstock-Guttman *et al*., 2000]. The anti-proliferative and apoptotic effects of IFNs discovered on different types of transformed cell lines and primary tumor cultures [Balkwill *et al*., 1989; Caraglia *et al*., 2005] account for their use in the clinical practice also as anti-tumor agents. As an example, the anti-proliferative effects of IFNs are successfully exploited in the clinics to treat hairy cell leukemia, chronic myeloid leukemia, multiple myeloma, melanomas, and also several subtypes of NHLs, including FL [Pfeffer *et al*., 1998], using these compounds alone or in combination with other drugs. It is worth mentioning how IFNs side effects are reversible and generally disappear a few days after the end of therapy [Bhatti et al., 2007]. Interestingly, IFNs potentiate retinoid-induced cell differentiation and act synergistically with these compounds to inhibit cell proliferation [Bollag *et al*., 1992]. Moreover, RA/IFN combination was previously shown to exert also pro-apoptotic effects in different cancer cell systems [Altucci et al., 2001]. In clinical trials, combination of IFN-α and 9-*cis*-RA gave excellent results in the treatment of squamous cell carcinoma of the skin and cervix, generating about two-fold higher response rates than those achieved with higher doses of either agent used alone. This two compounds, in particular, were used because of their synergistic inhibition of squamous cell carcinoma cell lines proliferation *in vitro* [Bollag *et al*., 1992]. Combination treatments between RA and IFNs have been used also in CTCL [Bollag *et al*., 1992]. Because the host immune response plays a pivotal role in the immune surveillance and clearing of CTCL tumor cells, cytokines such as IFN-α are employed as enhancers to stimulate antitumor immune responses [Lansigan *et al*., 2008]. Such extensive use of these cytokines for the treatment of human diseases underscored their therapeutic importance and

emphasized, over the years, the need to better understand the mechanisms by which they generate their pleiotropic biological effects.

The best characterized signal transduction cascade triggered by IFNs is the Janus tyrosin kinases (JAKs), Tyk, and Jak pathway. Being associated with the IFN receptor, these kinases are directly phosphorylated and activated following receptor binding. Once activated, they can in turn regulate the activation of different IFN-dependent transcription factors, such as the Signal Transducer and Activation of Transcription (STAT) proteins, which are involved in a great variety of cellular processes, including differentiation and apoptosis, and are essential for transcriptional activation of IFN stimulated genes (ISGs) [Platanias, 2003]. It is now well established that tyrosine 701 phosphorylation of STATs by activated JAKs is required for their nuclear translocation and binding to the promoter of ISGs. In addition, phosphorylation of STATs at serine 727, generally mediated by the PKCδ kinase [Uddin *et al*., 2002], is also required for its full transcriptional activity [Darnell *et al*., 1994]. Beyond the classic JAK-STAT pathways, evidence has emerged over recent years implicating other signaling cascades in the transmission of IFN signals. Among them, MAPK pathways appear to play key roles in the optimal transcriptional regulation in response to IFNs [Katsoulidis *et al*., 2005]. The mTOR/S6K pathway is also regulated by both type I and type II IFNs, and the downstream translational repressor 4E-BP1 exhibits a negative regulatory role in the generation of the antiviral effects of IFN-α, raising the possibility that this signaling cascade accounts for IFN-dependent mRNA translation [Kaur *et al*., 2007]. Conflicting evidence, however, emerged on the role of Akt in IFN-dependent signaling. Some studies have suggested that this kinase plays a negative role in generating IFN responses, by blocking IFN-dependent apoptosis [Yang *et al*., 2001; Lei *et al*., 2005]. On the other hand, other studies suggested that Akt is a positive regulator of IFN-stimulated adhesion of monocytes [Navarro *et al*., 2003]. More recently, Kaur *et al*. demonstrated that IFNs can rapidly activate Akt, and its activation is required for mRNA translation of ISGs and, ultimately, for the induction of its biological effects, with a complementary function to the IFN-activated JAK-STAT pathways. In fact, Akt activity in this case is unrelated to the regulatory functions on IFN-dependent STAT phosphorylation/activation or transcriptional regulation [Kaur *et al*., 2007]. Despite this result, most of available data support the existence of a cross-talk between the PI3-K/Akt pathway and STAT proteins in the signaling cascade triggered by IFNs. For example, inhibition of PI3-K abolishes IFNγ-induced phosphorylation of STAT1 at serine 727 [Nguyen *et al*., 2001], while STAT3 has an essential role in PI3-K induced oncogenic transformation [Hart *et al*., 2011].

The control of different signaling pathways by IFNs results in the ability of these cytokines to regulate a large number of genes, providing one explanation to their pleiotropic effects. Some of these genes are involved in the control of proliferation and apoptosis. For example, INFs variably regulate apoptotic cell death by both up-regulating pro-apoptotic genes, such as Bax and Bak, or down-regulating the pro-survival Bcl-2, Bcl-X_L, and Mcl-1 genes, often over-expressed in B-NHLs [Chawla-Sarkar *et al*., 2003]. Gene profiling experiments have implicated also the pro-apoptotic Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins Noxa among the ISGs [Sun *et al*., 2004], and it has been recently demonstrated that IFN regulatory factors (IRFs) are able to regulate Noxa expression following IFN treatment [Piya et al., 2011]. Results from gene expression profiling were subsequently confirmed by Sun *et al*., who showed a potent induction of Noxa mRNA and protein in multiple human tumor cell lines after exposure of these cells to IFN [Sun et al., 2005]. The mechanisms of Noxa-induced apoptosis mainly involve the intrinsic apoptotic pathway, where this protein contributes to disruption of mitochondrial outer membrane integrity. When cells receive intrinsic death signals, in fact, there is a subsequent transcriptional up-regulation and/or activation of pro-apoptotic BH3-only proteins such as Bim, Bid, Bad, Bmf, Noxa, and Puma. Pro-apoptotic proteins are able to bind anti-apoptotic members of the Bcl-2 family (for example, Bcl-2, Bcl-X_L, A1/Bfl1, and Mcl-1) and inhibit their activity. In addition to inhibiting anti-apoptotic proteins, some direct pro-apoptotic activators, including Bid and Bim, can also bind and activate the apoptotic effectors Bak and Bax. Once activated, Bak and Bax can change their conformation to form oligomers, which subsequently punch pores in the outer mitochondrial membrane, leading to its permeabilization and allowing the release into cytosol of pro-apoptotic proteins, such as cytochrome c (Cyto c) and second mitochondria-derived activator caspase (SMAC), , which initiate a cascade of caspase activation events ultimately resulting in apoptosis (Figure 4) [Zhang *et al*., 2013]. Notably, Noxa/Mcl-1 interaction plays a critical role in survival of several tumors, including MCL. Indeed, both Noxa up-regulating and BH3-mimetic drugs were shown to induce significant apoptotic responses in MCL [Perez-Galan et al., 2007]. A decade after its initial identification, Noxa was defined as a p53-inducible gene. Though p53 remains the main transcriptional regulator of this pro-apoptotic protein, recent studies have demonstrated a p53-independent induction of Noxa involving either E2F1 or p73 [Hershko *et al*., 2004; Flinterman *et al*., 2005]. Interestingly, also IFN-triggered Noxa up-regulation is independent of p53 but requires IFN-response pathways [Sun *et al*. 2005]. Finally, in the last years investigators demonstrated a role for FOXOs in activating Noxa transcription in neuronal cells [Obexer *et al*., 2007] and cancer cells [Valis *et al*., 2011].

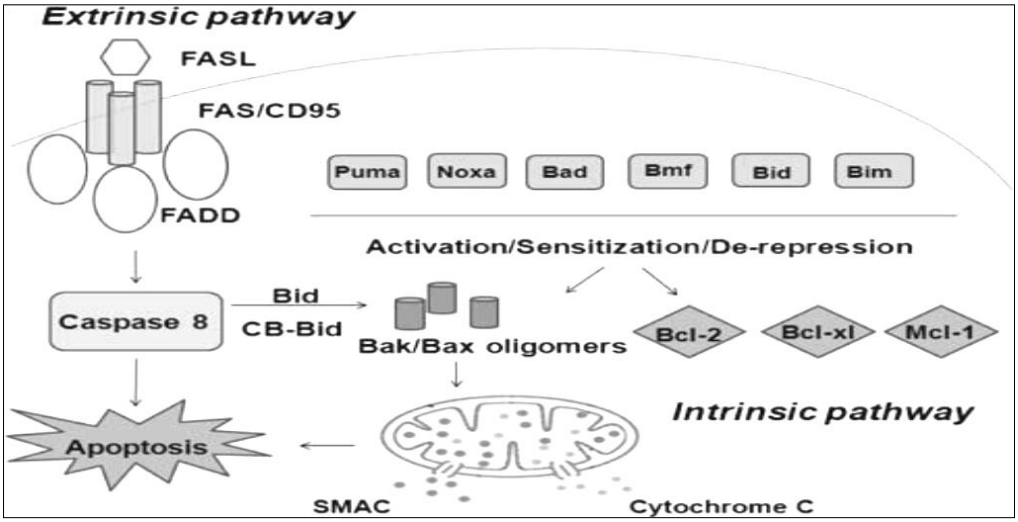


Figure 4. Two main pathways of apoptosis: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is triggered by many kinds of death signals from cell surface. FAS/FAS ligand (FASL) is an example. The intrinsic pathway is mainly regulated by BH3-only family members [Zhang *et al*., 2013].

Among the genes transcriptionally regulated by IFNs, another intriguing gene that has been shown to be involved in the apoptotic response of different cellular systems is the gene encoding for Phospholipid Scramblase 1 (PLSCR1) [Sahu *et al*., 2007]. Belonging to the flippase family proteins, PLSCR1 is a multiply palmitoylated endofacial plasma membrane protein originally identified based on its capacity to promote the exposure of phosphatidylserine (PS) on cell surface, a hallmark event of both the extrinsic and intrinsic apoptotic pathway's early stages, [Balasubramanian *et al*., 2007]. Accordingly to Zhao *et al*., IFN-α-induced PLSCR1expression seems to depend upon STAT1 activation through sequential induction of PKCδ and JNK kinases [Zhao *et al*., 2005]. As one of the most potently activated ISGs, PLSCR1 has been suggested to be involved in a broad spectrum of cellular responses to IFNs in the cytoplasm [Zhou *et al*., 2000]. Interestingly, when it fails to be palmitoylated, PLSCR1 can be imported also into the nucleus where it binds to genomic DNA, suggesting a potential role for this protein in regulating gene transcription [Chen *et al*., 2005]. Furthermore, PLSCR1 is involved also in protein phosphorylation and may be a potent and necessary activator of other genes in response to IFNs [Sahu *et al*., 2007]. Though the expression of PLSCR1 markedly increases especially in response to IFN-α, it has been seen to increase also after treatment with ATRA in different leukemia cell lines and, more interestingly, suppression of PLSCR1 expression by small interfering RNA inhibits ATRA-induced leukemic cell differentiation [Nakamaki *et al*., 2002; Zhao *et al*., 2004]. Notably, a recent work suggests that PLSCR1 could be a novel diagnostic biomarker and an important prognostic factor for colorectal cancer [Kuo *et al*., 2011], providing the rationale to

better characterize the potential role of this protein as a new tumor biomarker, by studying not only its association with clinical-phatological parameters and patients outcome, but also its possible direct involvement in mediating drugs therapeutic effect.

Considering that the most active RA isomer 9-*cis*-RA has been used in several clinical trials for different tumor histotypes [Miller *et al*., 1996], the evidence described so far provide the rational background to investigate its ability to induce apoptotic cell death in MCL and other types of NHLs when used in combination with IFNs, and in particular with the IFN most frequently used against hematologic malignancies, the IFN-α, and how this combination may potentially improve the management of these lymphoproliferative disorders.

AIMS

Constitutive activation of Akt is one of the most frequent alterations observed in human cancer, including hematologic malignancies [Drakos *et al*., 2008]. Being critical for growth and survival, tumor cells carrying an inherently activated Akt may depend on this kinase for proliferation and resistance to apoptosis. Research has also shown that alteration of the PI3-K/Akt pathway signaling translates into resistance to available treatments and a poor patient prognosis [Schatz, 2011]. These features make this multifunctional pathway a particularly attractive therapeutic target for patients with lymphoma, where Akt often appears deregulated. Molecular ties linked to abnormal PI3-K signaling pathway have been demonstrated in mantle cell lymphoma as well as in follicular and cutaneous T cell lymphoma [Schatz, 2011]. Enthusiasm related to the clinical expectations of currently used Akt inhibitors is currently dampened by the pronounced toxicity of available drugs. Therefore, more effective and safe therapeutic options targeting Akt are needed.

We previously demonstrated that retinoic acid induces a marked anti-proliferative response, but no apoptotic effects, in both cell lines and primary cultures derived from MCL patients [Guidoboni *et al*., 2005]. However, the sole growth inhibition of tumor cells cannot be considered a sufficient effect to finally eradicate the disease *in vivo*. On these grounds, taking into account the large body of evidence supporting the therapeutic potential of RA and IFNs combination in tumors, we planned to investigate if the combination of 9-*cis*-RA with IFN-α, whose triggered pathways show numerous functional interactions, is capable to enhance the anti-proliferative activity exerted by RA, and even to induce significant pro-apoptotic effects in MCL, FL, and CTCL cell lines, and to study the effects of the co-treatment on the expression of pro-apoptotic proteins potentially involved in 9-*cis*-RA/IFN-α-induced cell death. In particular, we focused our interest on Noxa and PLSCR1, two proteins involved in regulation/execution of the apoptotic process in other cellular systems [Sahu *et al*., 2007; Zhang *et al*., 2013]. We analyzed their possible function in inducing 9-*cis*-RA/IFN-α-dependent pro-apoptotic effect and sensitization to other anti-tumor drugs-mediated apoptosis in MCL through RNA-i*nterference* technology-based experiments. Moreover, considering that the PI3-K/Akt pathway is critical for survival of MCL, FL, and CTCL cells, and that Akt, but not mTOR inhibition, induces apoptotic responses in these NHLs histotypes [Dal Col *et al*., 2008; Marzec *et al*., 2008; Bhende *et al*., 2010] we also investigated the possible effects of RA/IFN-α treatment on their inherent PI3-K/Akt activation. Therefore, the present study was aimed at identifying key regulators of apoptosis of potential usefulness as new biomarkers of susceptibility/resistance to anti-tumor

therapies. To this end, we have characterized the molecular mechanisms underlying 9-*cis*-RA/IFN-α-dependent apoptosis, paying particular attention to the possible involvement of the PI3-K/Akt/mTOR pathway. This with the final goal to re-evaluate the relevance of 9-*cis*-RA/IFN-α combination as a potential alternative and relative toxic therapeutic strategy in the setting of aggressive NHLs characterized by inherent Akt activation.

MATERIALS AND METHODS

Patient samples

Four patients with MCL were identified on the basis of morphological, immunophenotypic and molecular criteria according to W.H.O. lymphoma classification (Table 1). The study was performed in accordance with protocols approved by the local IRB, and all patients gave their informed consent. Mononuclear cells were isolated from unicellular suspension obtained from mechanically minced lymph nodes or spleen. Enriched MCL samples (>70% MCL) were cryo-preserved in 10% DMSO until further study. Before use, samples were re-suspended in RPMI 1640 medium (Lonza) containing 10% fetal calf serum (FCS) and antibiotics.

Table 1. MCL cases

Case No.	*Sex/Age*	*Malignant cells (%)*	*Type*	*Cyclin D1*	*$p27^{Kip1}$*	*Sample analyzed*
MCL4	F/72	95%	Classical	+	NA	Lymph node biopsy
MCL5	M/50	86%	Classical	+	low	Lymph node biopsy
MCL6	M/64	95%	Classical	+	low	Lymph node biopsy
MCL7	M/72	96%	Classical	+	low	Spleen biopsy

The expression of cyclin D1 and $p27^{Kip1}$ proteins was detected by immunohistochemistry. (NA: not available)

Cell lines

Mino, SP53, and Jeko-1 cell lines were generously contributed by Dr. Raymond Lai, Canada. Granta 519 was purchased by DSMZ (Braunschweig, Germany). DOHH2 and HF4 cell lines were generously contributed by Dr. Filippo Belardelli, Rome, while HUT78 cell line by Dr. Valter Gattei, Aviano. Cell lines were authenticated by fingerprinting (Power Plex 1.2, Promega) in January 2011. Granta 519 were cultured in medium Dulbecco's modified Eagle medium (DMEM, Lonza) supplemented with 10% serum fetal bovine (FBS) heat-inactivated (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM L-glutamine. For all the other cell lines DMEM was replaced with RPMI 1640 (Lonza). All cells were maintained in culture under the following conditions: 95% O2, 5% CO2, 98% humidity and 37 °C.

Proliferation assay, caspase activity, and apoptosis detection

Cell proliferation was evaluated by ^{3}H-thymidine uptake and caspase 8, 9, and 3 activation using a fluorimetric commercial kit (Immunochemistry Technologies). Apoptosis was evaluated by Annexin V/Propidium iodide (PI) stains and/or by active/cleaved caspase 3 analysis. Several apoptosis evaluations were made also with ImageStreamX technology by DRAQ5 dye. In this case, samples were acquired with the ImageStreamX instrument (Amnis Corporation, Seattle, WA) and data analyzed using the INSPIRE software. This new technology allows distinguishing between viable and apoptotic cells on the basis of their nuclear morphology [Rieger *et al*., 2010]. All flow cytometric analyses were performed on a FC500 flow cytometer (Beckman Coulter).

Quantitative real-time PCR analysis

Total RNA was extracted from 1 to 3 x 10^{6} cells by QIAGEN RNeasy Mini Kit. 1µg of RNA was retro-transcribed into cDNA using the ISCRIPT RT OneTube Supermix according to manufacturer's instructions (BIO-RAD, Hercules, CA, US). Real-time PCR was performed in a Thermal Cycler CFX96 (BIO-RAD), using SsoFast EvaGreen Supermix (BIO-RAD, Hercules, CA, US). The specific primers were designed by Primer3 Input software (version 0.4.0), and were synthesized by SIGMA-Aldrich Co. (St Luois, Missouri, US). Primers used for $p21^{WAF1/Cip1}$: forward: 5'-CCTGGCACCTCACCTGCTCT-3', reverse: 5'-AGAAGATCAGCCGGCGTTTG-3'. Primers used for PLSCR1: forward: 5'-AAATCCAAGCTCCTCCTGGT-3', reverse: 5'-TTTGCCAACCACACACTGTT-3'. Four different housekeeping genes, β-actina (forward: 5'-AGAGCTACGAGCTGCCTGAC-3', reverse: 5'-AGCACTGTGTTGGCGTACAG-3'), GAPDH (forward: 5'-GGAACGGTGAAGGTGACAGC-3', reverse: 5'-TCACCTCCCCTGTGTGGACT-3'), β2-microglobulin (β2M), and 18-S were used. Specific primers for 18-S and β2M were kindly provided by BIO-RAD, while the other primers where designed by the software, as above. Normalized relative quantity was calculated with "ΔΔCt" method.

Antibodies and reagents

Bcl-xL, A1/Bfl-1, Mcl-1, Bax, Bid (Rabbit), Puma, phospho-Akt (Ser473), phospho-S6rp (Ser235/236), phosphor-mTOR (Ser2448), phospho-FOXO3a (Ser318/321), Akt, phospho-STAT1(Tyr701), phospho-STAT1(Ser727), STAT1, and cleaved caspase 3 (D175) antibodies were from Cell Signaling Technology; Noxa, PLSCR1, Mcl-1 (Y37), Bcl-2A1 (EP517Y) antibodies were from Abcam; $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ from BD Transduction Laboratories; β-tubulin (H-235), PARP (F2), S6rp, cyclin D1 (DCS-6), $p57^{Kip2}$, $p45^{Skp2}$ and Cks1 from Santa Cruz

Biotechnology; Bid (Mouse) antibody from SoutherBiothec; Bak (N-terminal) and FOXO3a antibodies from Millipore; and FITC-conjugated CD20 (L27) mouse-monoclonal antibody from BD Bioscience (Franklin Lakes, NJ USA). Vital nuclear dye DRAQ5, SH5 (Akt inhibitor) and LY294002 (PI3-K) were purchased from Alexis Biochemicals; rapamycin (mTORC1 inhibitor), G418, and 9-*cis*-RA from Sigma, and IFN-α (IntronA) was purchased from SP Europe. 9-*cis*-RA and IFN-α were used at 1 μM and 1000U/mL, respectively. Doxorubicin, bortezomib, and rituximab were from EBEWE, JANSSEN-CILAG, and ROCHE respectively, and were used at the concentrations of 50 nM, 5 nM, and 10 μg ml-1 respectively.

Extract preparation, immunoprecipitation, and Western Blot analysis

Whole cell lysates were prepared in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 2 mmol/L sodium orthovanadate, 25 mmol/L h-glycerophosphate, 25 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 Amol/L okadaic acid, 5 Ag/mL leupeptin, 5 Ag/mL aprotinin, 0.2% Triton X-100, and 0.3% NP40] and lysed for 30 minutes on ice. Total protein extracts were obtained by centrifugation at 13,000 rpm for 15 minutes and protein concentration was determined by the Biorad Bradfor Protein Assay (Milan, Italy). Proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblotting was performed using the enhanced chemiluminescence plus detection system (PerkinElmer) through Chemidoc XRS$^+$ instrument (Biorad). In immunoprecipitation studies, 500 to 1,000 μg of proteins were incubated with 1 μg of appropriate antibody and 50 μL of protein A-Sepharose CL4B (Amersham International) overnight, centrifuged, and washed thrice with lysis buffer. Proteins were eluted with Laemmli sample buffer and fractionated using SDS-PAGE, and Western blots were performed.

Intracellular flow cytometry

Cells (10^6 per sample) were fixed with 2% of paraformaldehyde at room temperature for 10 minutes, then permeabilized with 500 μL of cold methanol and incubated with the primary antibodies for 1 hour or overnignt at 4°C. After two washes with PBS containing 0.5% bovine serum albumin (BSA), cells were incubated for 30 minutes at 4°C with PE-anti-Rabbit secondary antibody and analyzed by flow cytometry.

Multispectral imaging flow cytometry

The ImageStreamX technology allows for multi-spectral imaging of cells in flow and combines the analysis of morphometric features with fluorescence staining. For protein co-localization

experiments, each sample was labelled with Mcl-1 or A1/Bfl-1 antibodies and Bid antibody and the vital nuclear dye DRAQ5. The staining of the nucleus allowed the distinction between viable and apoptotic cells on the basis of the nuclear morphology, excluding thus apoptotic cells from the analysis. Cells double-positive for both Mcl-1 and Bid or A1/Bfl-1 and Bid were selected and compared using an algorithm of the IDEAS analysis software which calculates the degree of co-localization through the Bright Details Similarity Score (SBDS). To assess the lower limit of SBDS, a control sample was labelled with antibodies against Mcl-1 and PARP, two proteins localized in different cellular compartments, and the value calculated was 0.3786 ± 0.1254 (SBDS ± SD). In addition, the SBDS due to a non-specific overlap of two proteins localized in the same intracellular compartment but not associated with each other was assessed by analyzing cells labelled with antibodies against Bid and tubulin, both localized into the cytoplasm, 1.668 ± 0.37 (SBDS ± SD). Therefore, we considered true co-localized events those having SBDS values of 2.25 or greater. Moreover, considering that RA/IFN-α-treatment also induced A1/Bfl-1 and Bid down-regulation, to exclude that the RA/IFN-α-induced decrease of the SBDS could be merely due to the decrease of the expression of these proteins, we selected for analysis only double-positive cells showing a fluorescence intensity comparable to that of untreated cells.

In FOXO3a nuclear localization experiments, each sample was labelled with an antibody against FOXO3a and the nuclear dye DRAQ5. Using an algorithm of the IDEAS analysis software, the Similarity Score (SS) between FOXO3a and DRAQ5 staining was calculated for each sample. To define the range of SS variability, the lower and the upper limits were calculated. To this end, the first control sample was labelled with DRAQ5 and an antibody against tubulin (cytoplasmic marker) and the score value was -1.316 ± 0.5538 (SS ± SD). The second control sample was labelled with DRAQ5 and an antibody against PARP (nuclear marker) and the score value was 2.426 ± 0.4956 (SS ± SD). All events showing a positive SS were considered with high similarity between FOXO3a and DRAQ5, thus indicating a nuclear localization of FOXO3a. Only viable cells were selected on the basis of morphologic features.

Bid-Mcl-1 and Bid-A1/Bfl-1 co-localization and FOXO3a nuclear internalization

Cells (10^6 per sample) were fixed, permeabilized and labelled as described above. Samples were acquired with the ImageStreamX (Amnis) using the INSPIRE software. For co-localization experiments, samples were labelled with Mcl-1 or A1/Bfl-1 antibodies and Bid antibody and DRAQ5. Then, cells double-positive for Mcl-1 and Bid or A1/Bfl-1 and Bid were selected and compared using an algorithm of the IDEAS software which calculates

the specificity and the degree of the fluorescence signals co-localization through the Similarity Bright Details Score (SBDS) [Beum *et al*., 2006].

For the analysis of FOXO3a nuclear internalization, samples were labelled with an antibody against FOXO3a (1:100) at 4°C overnight. Then, cells were stained with the PE-anti-Rabbit secondary antibody and DRAQ5. Using an algorithm of the IDEAS software, the Similarity Score (SS) [George *et al*., 2006] between FOXO3a and DRAQ5 staining was calculated for each sample.

Phospho-STAT1 nuclear internalization

10^6 cells per sample were fixed and permeabilized as described above, and incubated with an antibody against phospho-STAT1(Y701) (1:30) at 4°C overnight. After two washes with PBS/0.5% BSA, cells were incubated for 30 minutes in ice with PE-anti-Rabbit secondary antibody. After other two washes the DRAQ5 nuclear dye was added and then cells were acquired with the ImageStreamX instrument (Amnis) using the INSPIRE software. Only viable cells were selected on the basis of morphologic features, and only phospho-STAT1 positive cells were analyzed. Using an algorithm of the IDEAS analysis software [George *et al*., 2006], the Similarity Score (SS) between phospho-STAT1 and DRAQ5 staining was calculated for each sample. To define the range of variability of the SS, the lower and the upper limits were calculated as described above.

MAPK Transcription Factors Assay

To quantify the DNA-binding activity of MAPK-regulated transcription factors ATF-2, c-Jun, c-Myc, MEF2, and STAT1 we used the TransAM™ MAPK Family Transcription Factor assay Kit (Active Motif®), which combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. Nuclear extracts of the samples were prepared according to manufacturer's recommendations. Absorbance was read at 450 nm on the Microplate Autoreader system (BIO-TEK INSTRUMENTS).

Noxa and PLSCR1 silencing

For silencing of Noxa gene or PLSCR1 gene two different shRNA PMAIP1 (phorbol-12-myristate-13-acetate-induced protein 1) or four shRNA PLSCR1 constructs, respectively, were obtained by sub-cloning the double-stranded 64-mer oligonucleotide, containing the PMAIP1 or the PLSCR1 target sequences, into the pSUPER.retro.neo+GFP vector (pSUPER; OligoEngine). Infectious supernatant from pSUPER and pSUPER.retro-shPMAIP1 or pSUPER.retro-

shPLSCR1 retrovirally transfected Phoenix cells were collected after 48 hours and used for three cycles of infections [Becknell *et al.*, 2005]. Upon infection, cells were selected with G418 (1 mg/mL) and the infection efficiency was checked through the detection of GFP expression by flow cytometry (97% GFP-positive cells). Different clones of infected cells were then obtained after seeding cells in 96-well plate at an initial density of 25 cells/well in 200 µL of medium supplemented with G418. Target sequences used for silencing were:

- two for Noxa (PMAIP1): A. 5'-AAACTGAACTTCCGGCAG-3'; B. 5'-TCTGATATCCAAACTCT-3';
- four for PLSCR1: A. 5'-GGACCTCCAGGATATAGTG-3'; B. 5'-CTCTGGAGAGACCACTAAG-3'; C. 5'-AGTCTCCTCAGGAAATCTG-3'; and the mismatched sequence MIS: 5'-GGACGTCCTGGATTTAGTG-3.

Immunoblotting analysis of transfected Phoenix cells identified the construct shPLSCR1A as the most efficient in protein silencing, therefore we selected this to perform all subsequent experiments.

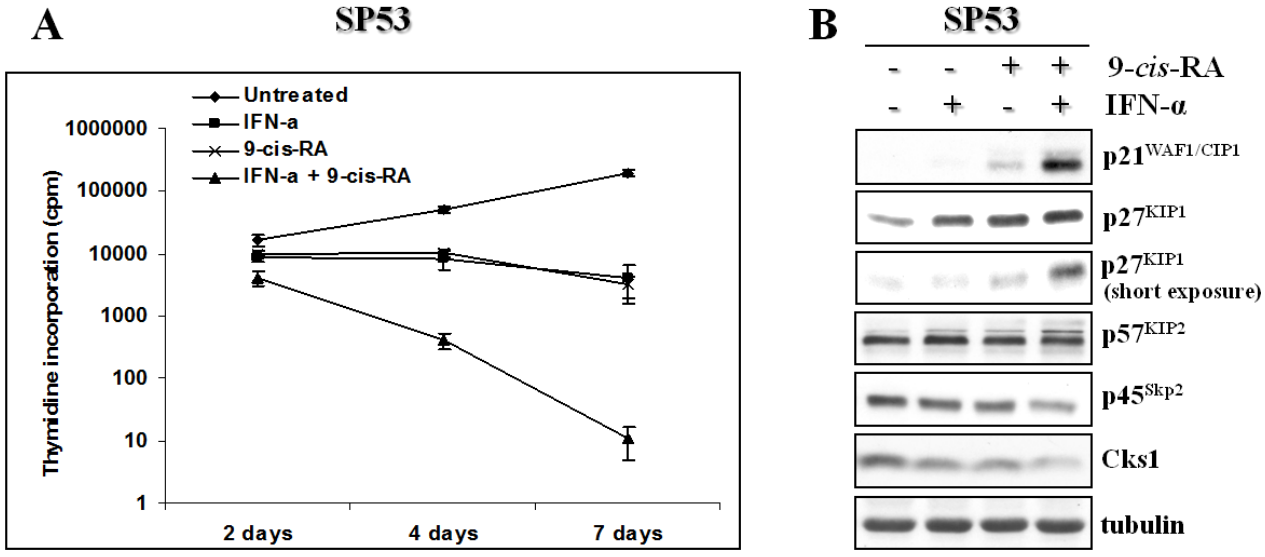
Immunostaining and flow cytometry analysis of CD20 expression.

Direct immunofluorescence staining was performed with fluorescein isothiocyanate conjugated anti-CD20 (clone L27) mouse-monoclonal antibody. Cells were prepared for flow cytometry according to standard methods. Briefly, $5x10^5$ cells per sample were collected and labeled with 5µL of monoclonal antibody in the presence of 10% rabbit serum, in a final volume of 100µL. Appropriate directly conjugated isotypic antibody (MsIgG1-clone X40; BD Bioscience) was used as non-specific staining control for all experimental conditions. All studies were performed using a FC500 flow cytometer (Beckman Coulter, Milan, Italy). For each sample $20x10^3$ cells were acquired and CD20 expression was measured as the mean fluorescence intensity (MFI).

RESULTS

1. Interferon-α significantly enhances the anti-proliferative activity exerted by retinoic acid in MCL cells.

Previous studies from our group demonstrated how 9-*cis*-RA, a pan-RAR and -RXR agonist, is the isomer with the strongest anti-proliferative activity against MCL cells [Guidoboni *et al*., 2005]. The combination of 9-*cis*-RA (1μM) with IFN-α (1000U/mL) for 2, 4, and 7 days resulted in an additive effect, with a more pronounced inhibition of SP53 MCL cell line growth as compared with cells treated with RA alone (Figure 5A). Accordingly, 9-*cis*-RA/IFN-α co-treatment increases the number of MCL cells in G_0/G_1 phases at the expenses of those in the S phase (not shown), suggesting a likely involvement of key molecules for G_1 to S phase transition. In particular, IFN-α enhances the $p27^{Kip1}$ protein up-regulation induced by 9-*cis*-RA as a result of a more pronounced inhibition of $p45^{Skp2}$ and Cks1, two SCF^{Skp2} ubiquitin-ligase complex components that are required for proteasome-dependent $p27^{Kip1}$ degradation (Figure 5B). Furthermore, immunoblotting analysis showed a 9-*cis*-RA/IFN-α-dependent up-regulation of $p21^{WAF1/Cip1}$, detected in MCL cell lines with either wild type (Granta 519, SP53) or mutated (Jeko-1, Mino) p53, so excluding a p53-only-dependent effect (Figure 5B and 5C). Consistently with a mainly post-translational effect, quantitative real-time PCR experiments showed no or only limited effects on the mRNA levels of $p21^{WAF1/Cip1}$ (not shown). More interesting, SP53 and Mino cells showed a marked down regulation of cyclin D1 protein levels after 5 days of co-treatment but not after the exposure to each drug alone (Figure 5D). These finding indicate that IFN-α enhances the anti-proliferative activity exerted by RA in MCL cells by decreasing the protein levels of cyclin D1 and further up-regulating $p27^{Kip1}$ and $p21^{WAF1/Cip1}$.



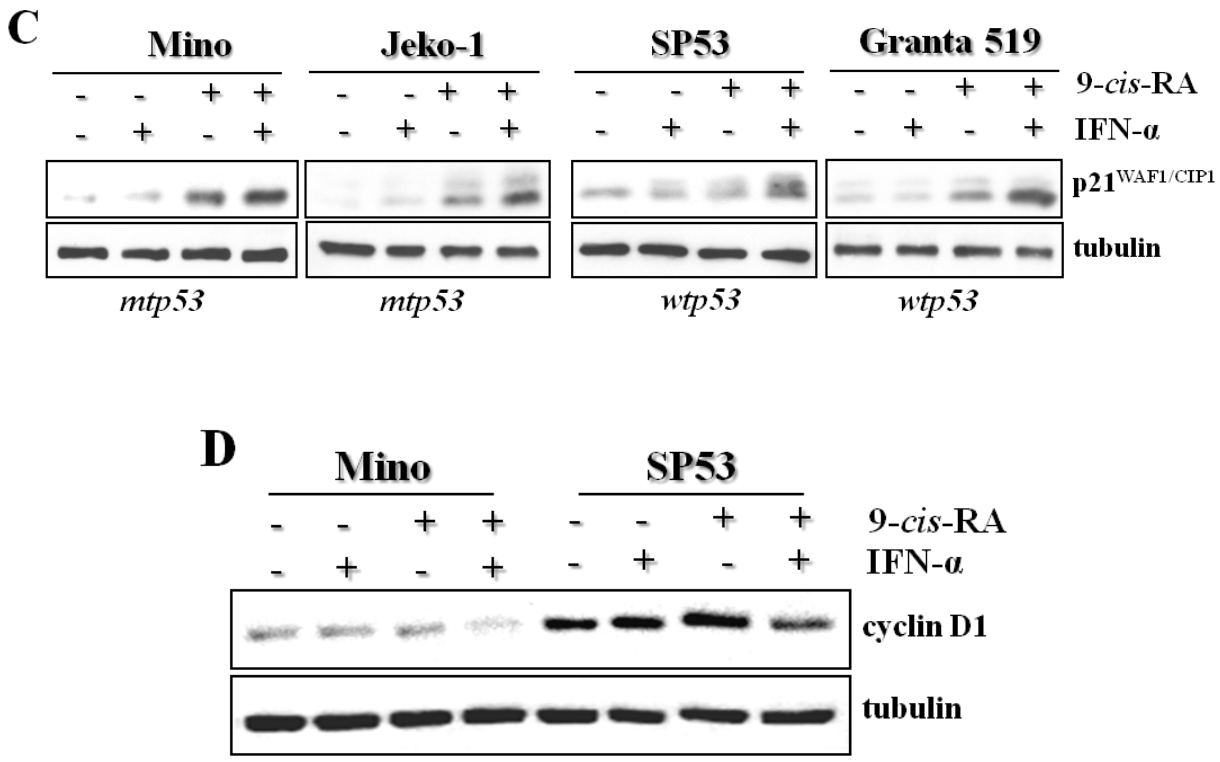
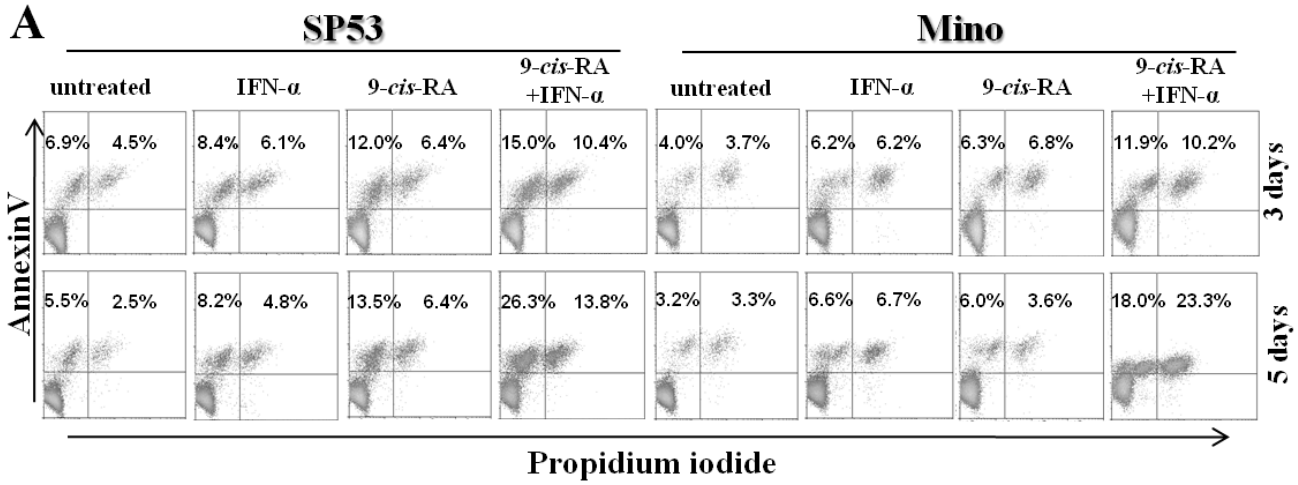


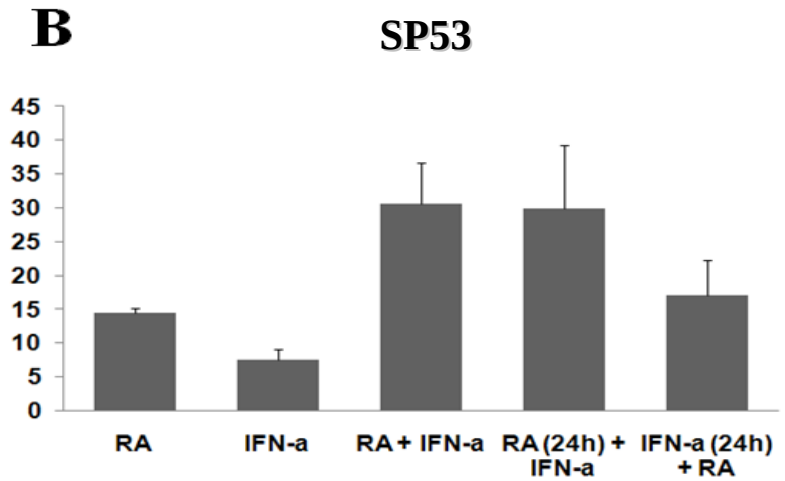
Figure 5. ***A)*** **IFN-α enhances the anti-proliferative activity exerted by 9-*cis*-RA in MCL cells.** DNA synthesis was assessed in SP53 cells by [^{3}H] thymidine incorporation after 6 hours. Points, mean from triplicate wells; bars, SD. The results are representative of one of three experiments. ***B)*** **9-*cis*-RA/IFN-α up-regulate p27^{Kip1} and p21$^{WAF1/Cip1}$ in SP53 cells** (3 days of treatment) ***C)*** **9-*cis*-RA/IFN-α-induced p21$^{WAF1/Cip1}$ up-regulation is not a p53-only-dependent event**. p21$^{WAF1/Cip1}$ up-regulation is detected in MCL cell lines with wild-type (SP53, Granta 519) and mutated (Mino, Jeko-1) p53 (3 days of treatment). ***D)*** **9-*cis*-RA/IFN-α combination down-regulates cyclin D1 expression in SP53 and Mino cells** (5 days of treatment).

2. 9-*cis*-RA sensitizes MCL cells to the caspase-dependent pro-apoptotic effect of IFN-α.

Although able to significantly slow down the proliferation of MCL cell lines, 9-*cis*-RA alone does not promote relevant apoptotic responses. [Guidoboni *et al.*, 2005]. Given the ability of IFN-α to cooperate with RA in inhibiting MCL cell growth, we also explored the possible induction of pro-apoptotic effects. To this end, sequential treatment experiments were conducted on SP53 and Mino cell for 3 and 5 days, and apoptosis was evaluated using AnnexinV/PI staining. RA/IFN-α combination induced more pronounced apoptotic effects in both MCL cell lines as compared with single treatments (Figure 6A) and, in particular, a 24-hours pre-treatment with 9-*cis*-RA sensitized MCL cells to the pro-apoptotic effect of IFN-α, whereas the reverse induced only modest effects (Figure 6B). The contribution of initiators and effectors caspases in 9-*cis*-RA/IFN-α-induced apoptosis was investigated in SP53 and Mino cells using specific fluorimetric caspase assays. Time-course experiments showed that both caspase 8 and 9 are

activated, almost simultaneously, after 36 hours of 9-*cis*-RA/IFN-α treatment (not shown). Immunoblotting analysis of several proteins of the Bcl-2 and BH3-only families, whose expression levels are essential for mitochondrial integrity, revealed a marked down-regulation of anti-apoptotic proteins such as Bcl-xL and A1/Bfl-1 by 9-*cis*-RA/IFN-α co-treatment, concomitantly with the presence of cleaved caspase 3, a known marker of ongoing apoptosis (not shown). Notably, the levels of the full-length Bid protein significantly decreased as likely consequence of its activation by caspase-dependent cleavage (not shown). Furthermore, although the expression levels of Bak and Bax pro-apoptotic proteins were comparable in untreated and treated cells, flow cytometry analysis using antibodies specific for their N-terminal domains revealed conformational changes indicating Bak and Bax activation only in cells exposed to 9-*cis*-RA/IFN-α (3 and 5 days) (Figure 6C and 6D). Taken together, these results indicate that 9-*cis*-RA/IFN-α combination triggers both mitochondrial/intrinsic and death receptor/extrinsic apoptotic pathways and promotes the shift of the critical balance between anti- and pro-apoptotic proteins in favor of apoptotic machinery activation.





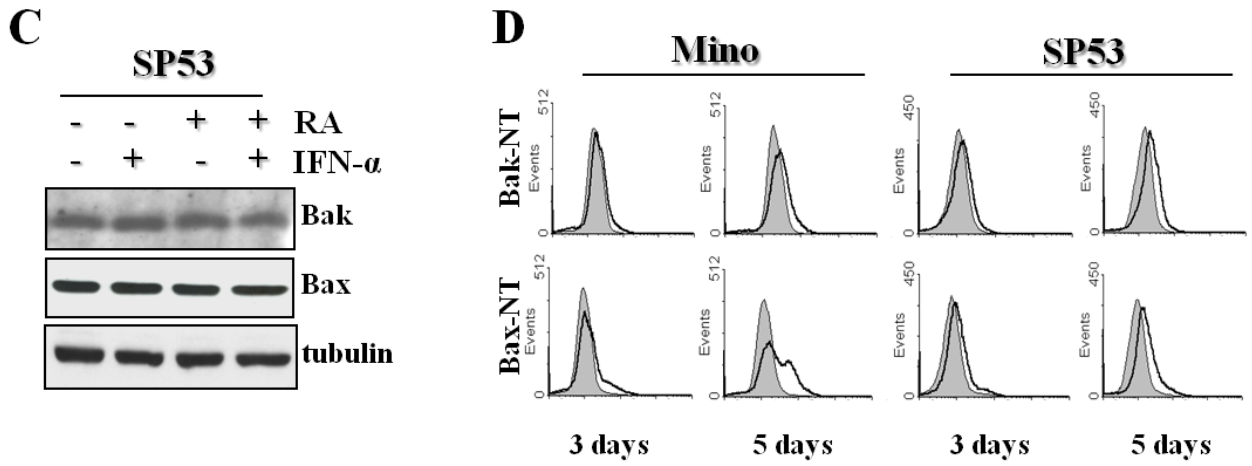
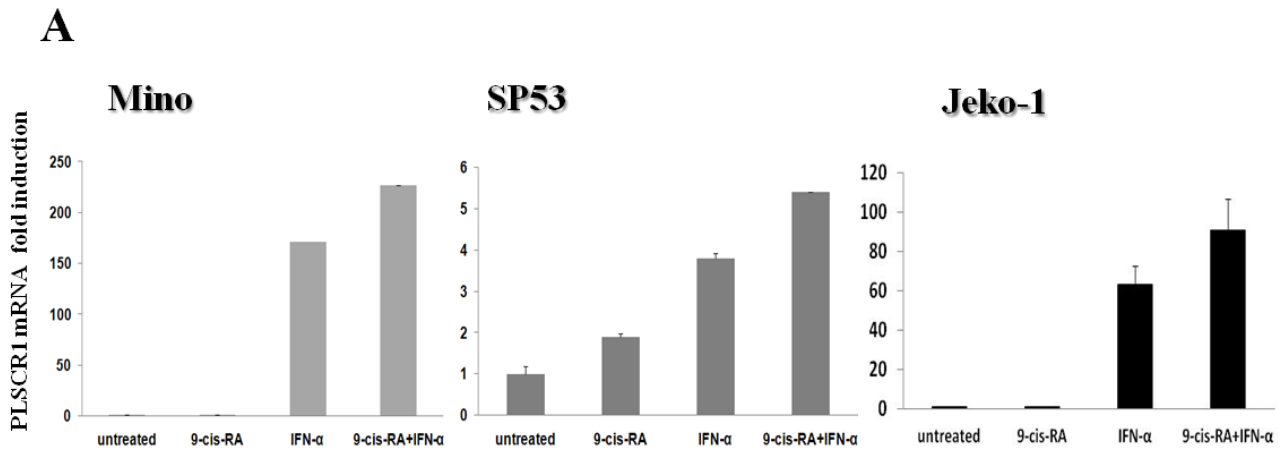


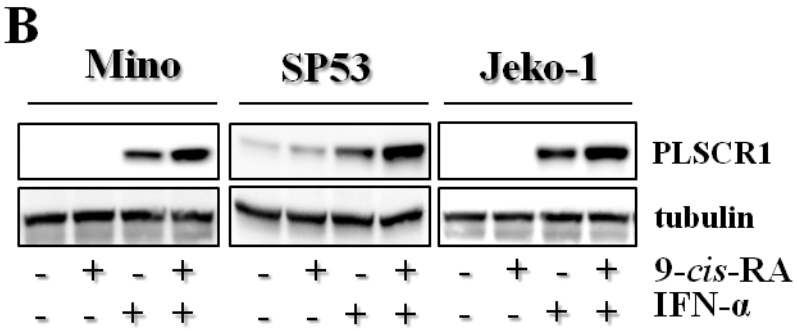
Figure 6. ***A)*** **Pro-apoptotic effects induced by 9-*cis*-RA/IFN-α in SP53 and Mino cells.** Data are representative of one of three independent experiments. ***B)*** **9-*cis*-RA sensitizes MCL cells to the apoptosis triggered by IFN-α.** SP53 cells were sequentially treated with 9-*cis*-RA (or IFN-α) for 24 hours and then IFN-α (or 9-*cis*-RA*)* was added. Apoptosis was evaluated after 3 days. Results are reported as percentage of increment relative to the control (untreated sample). Bars, mean from three independent experiments; error bars, SD. ***C)*** and ***D)*** **9-*cis*-RA/IFN-α treatment does not increase Bak and Bak expression levels but induces their activation.** SP53 total-cell lysates (50 μg) were analyzed by immunoblotting for Bak and Bax. Mino and SP53 cells were treated or not with 9-*cis*-RA/IFN-α for 3 and 5 days and stained with antibodies specific for the N-terminus domain of Bak or Bax, and for active caspase 3. Data reported are representative of one of three independent experiments.

3. Noxa and PLSCR1 are two critical players of 9-*cis*-RA/IFN-α-induced MCL apoptosis.

A previous microarray-based screening carried out to identify genes transcriptionally modulated by 9-cis-RA/IFN-α in SP53 cells, allowed the identification, among the genes significantly up-regulated by the treatment, two genes coding for proteins known to be involved in apoptotic responses in different cellular systems: Noxa and PLSCR1 (not shown). The transcriptional induction of PLSCR1 was confirmed by quantitative real-time PCR experiments in SP53, Jeko-1, and Mino cell lines. The results showed that treatment with IFN-α for 24 hours increased PLSCR1 mRNA levels in all three cell lines, and, more interesting, 9-*cis*-RA strengthened PLSCR1 induction when added to IFN-α, whereas it had no effect on PLSCR1 transcription if used alone (Figure 7A). Immunoblotting analysis confirmed a corresponding increase in PLSCR1 protein levels after the treatment and showed that the basal expression levels of this protein are heterogeneous in the three cell lines studied, with detectable levels only in SP53 cells (Figure 7B). With regard to the BH3-only protein Noxa, analysis of its expression disclosed a marked up-regulation especially in 9-*cis*-RA/IFN-α-co-treated cells (Figure 7C) and, more interestingly, Noxa and concomitant caspase 3 activation were significantly up-regulated by 9-*cis*-RA/IFN-α treatment also in 4 primary MCL cultures (Figure 7D). This effect seems to be

specific for lymphoma cells, since 9-*cis*-RA/IFN-α did not up-regulate Noxa, nor exerted any pro-apoptotic activity in normal B lymphocytes obtained from 2 different donors (not shown). To assess their potential role in 9-cis-RA/IFN-α-dependent apoptosis in MCL, we knocked-down Noxa or PLSCR1 expression in Mino cells using a short hairpin RNA (shRNA) viral expression vector containing a specific sequence targeting Noxa or PLSCR1 mRNA (pSUPER.retro-shPMAIP1 or pSUPER.retro-shPLSCR1 respectively). This approach allowed the generation of stably infected cells with a significant reduction of proteins up-regulation upon treatment, as verified by immunoblotting analysis (Figure 7E and 7F). In contrast, Mino cells infected with the empty vector (pSuper) did not show any detectable change in the expression levels of the proteins after the exposure to the treatment (Figure 7E and F). Notably, silencing of Noxa or PLSCR1 reduced the extent of apoptosis induced by 9-cis-RA/IFN-α treatment, as shown by PARP and caspase 3 cleavage, and the detection of apoptotic cell percentage by Annexin V/7AA-D staining (Figure 7E and 7G). Overall, these experiments indicate that PLSCR1 and Noxa up-regulation are both involved in mediating the pro-apoptotic activity of 9-cis-RA/IFN-α combination in MCL.





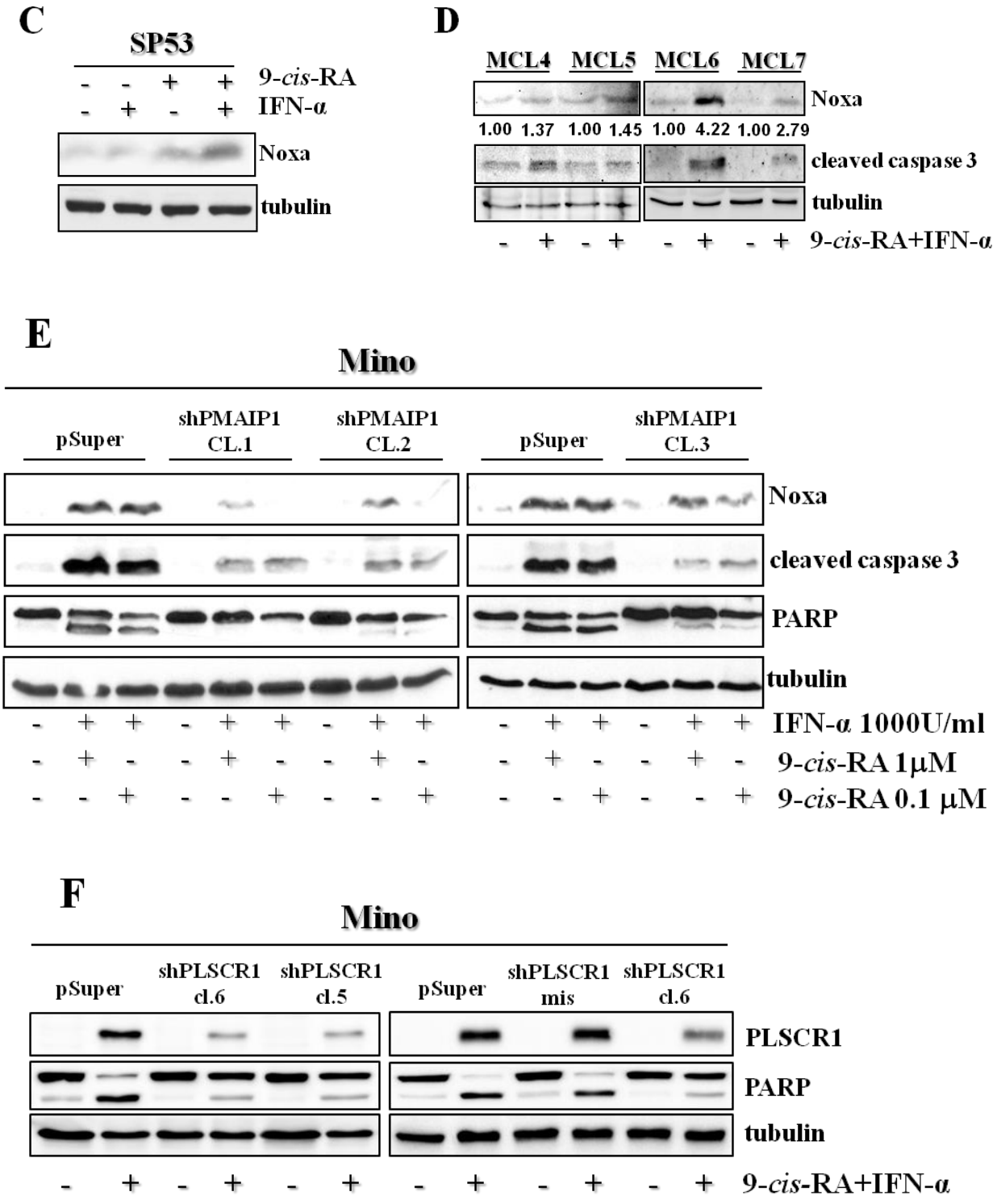
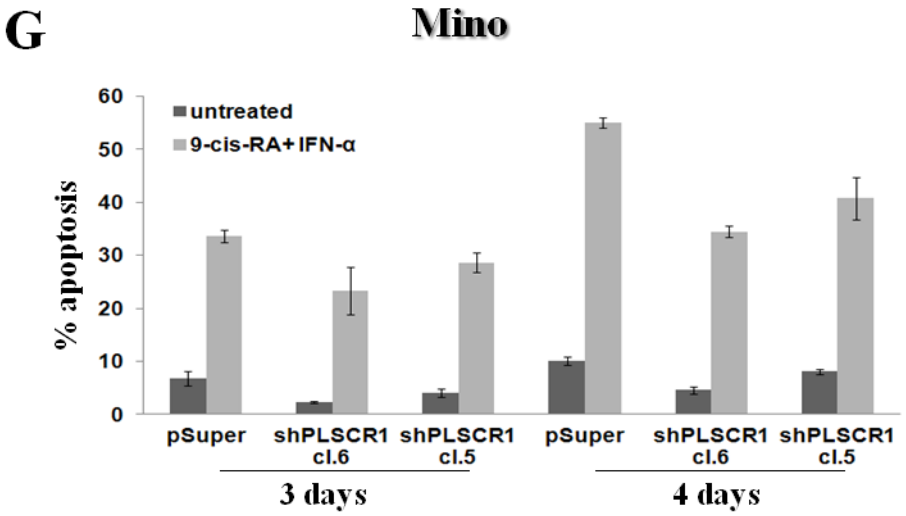


Figure 7. ***A)*** and ***B)*** **9-*cis*-RA significantly enhances the IFN-α-induced PLSCR1 up-regulation in MCL cells.** ***A)*** PLSCR1 mRNA was evaluated by real-time PCR in SP53, Mino, and Jeko-1 cells after 24 hours of treatment. One representative of three independent experiments is shown as mRNA fold induction. Bars, mean from three replicates; error bars, SD. ***B)*** Total-cell lysates (50 μg) were analyzed for the detection of PLSCR1. ***C)*** **9-*cis*-RA/IFN-α treatment modulates the BH3-only protein Noxa expression.** Total-cell lysates (50 μg), 3 days of treatment. ***D)*** **9-*cis*-RA/IFN-α combination induces Noxa up-regulation associated with caspase 3 activation in primary MCL cultures.** Purified primary lymphoma cells from 4 different MCL patients were treated with 9-*cis*-

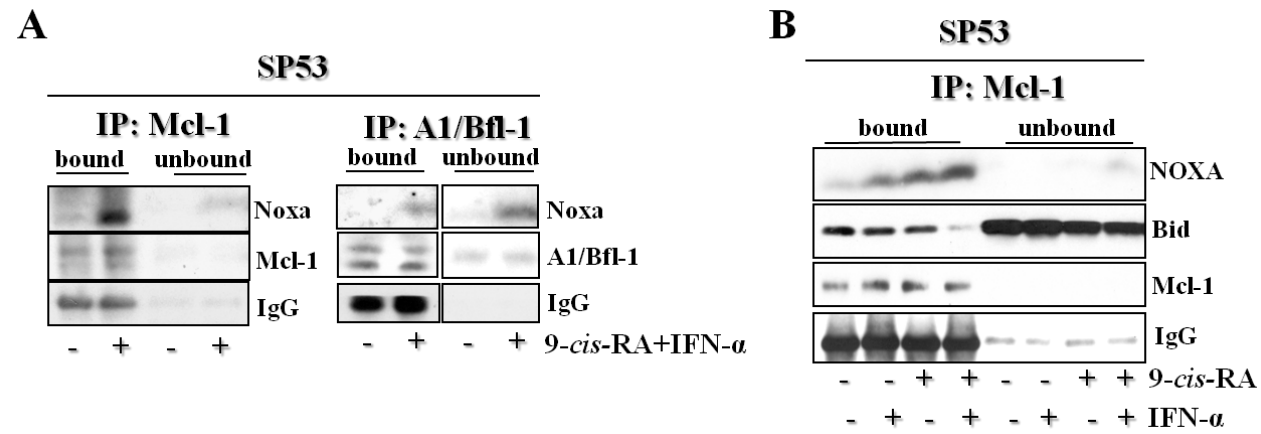
RA/IFN-α for 48 hours. Total-cell lysates (30 μg) were analyzed by immunoblotting for Noxa and cleaved caspase 3. The extent of 9-*cis*-RA/IFN-α-induced Noxa up-regulation is indicated in arbitrary units assigning to each untreated sample the value of 1.00. ***E)*** **Noxa knock-down reduces 9-*cis*-RA/IFN-α-dependent apoptosis.** Mino cells infected with empty vector (pSuper) and 2 different clones (CL.1 and CL.2) of cells infected with vector containing shPMAIP1 sequence A and 1 clone (CL.3) with the sequence B (see methods) were treated with 9-*cis*-RA/IFN-α for 3 days. Total-cell lysates (50 μg) were analyzed by immunoblotting for Noxa, cleaved caspase 3, and PARP. ***F)*** and ***G)*** **Silencing of PLSCR1 reduces the extents of apoptosis induced by 9-*cis*-RA/IFN-α treatment.** ***F)*** Total-cell lysates (50 μg) were obtained from Mino cells infected with empty vector (pSuper) and with vector containing shPLSCR1 target and mismatched sequences (cl.5, cl.6 and mis) (see methods) after 4 days of treatment. ***G)*** The percentage of apoptotic cells was detected by Annexin V/7AA-D stains and flow cytometric analysis. Bars, mean from three independent experiments; error bars, SD.



4. 9-*cis*-RA/IFN-α-dependent Noxa up-regulation allows Bid displacement from anti-apoptotic proteins in MCL cells.

Considering the ability of Noxa to specifically bind and consequently inactivate the anti-apoptotic Mcl-1 and A1/Bfl-1 proteins, the interactions between Noxa and these two Bcl-2 family members were investigated. Most of 9-*cis*-RA/IFN-α-induced Noxa co-immunoprecipitated with Mcl-1, the remaining amount being associated to A1/Bfl-1 (Figure 8A). Furthermore, the sequestration of Mcl-1 by up-regulated Noxa results in the displacement of the full-length Bid protein from Bid-Mcl-1 complexes (Figure 8B), allowing thus the consequent Bid activation through enzyme cleavage. The resulting truncated-Bid may thus directly contributes to the activation of the Bak and Bax apoptotic effectors. Taking advantage from multi-spectral imaging flow cytometry that allows for multi-spectral imaging of cells in flow, we analyzed the co-localization between Bid and Mcl-1 and A1/Bfl-1 also *in vivo* (see

materials and methods). To this end, we set up a protocol in which the cells were stained with specific antibodies to Mcl-1 or A1/Bfl-1 and Bid proteins and then the Bid-Mcl-1 and Bid-A1/Bfl-1 co-localization was analyzed only in double-positive live cells. As shown in Figures 8C and 8D, the SBDS detected in untreated samples was 2.48 ± 0.42 for Bid-Mcl-1 and 2.45 ± 0.49 for Bid-A1/Bfl-1 and in both cases the score significantly decreased when the cells were treated for 3 days with 9-*cis*-RA/IFN-α. Moreover, in treated samples, the percentage of cells showing a significant co-localization of the two proteins (with SBDS 2,25) was reduced from 72% to 19.2% for Bid-Mcl-1, and from 66.4% to 17.2% for Bid-A1/Bfl-1. These results indicate that the treatment induces the displacement of Bid from Bid-Mcl-1 and Bid-A1/Bfl-1 complexes through Noxa up-regulation and this event precedes and promotes the apoptotic process.



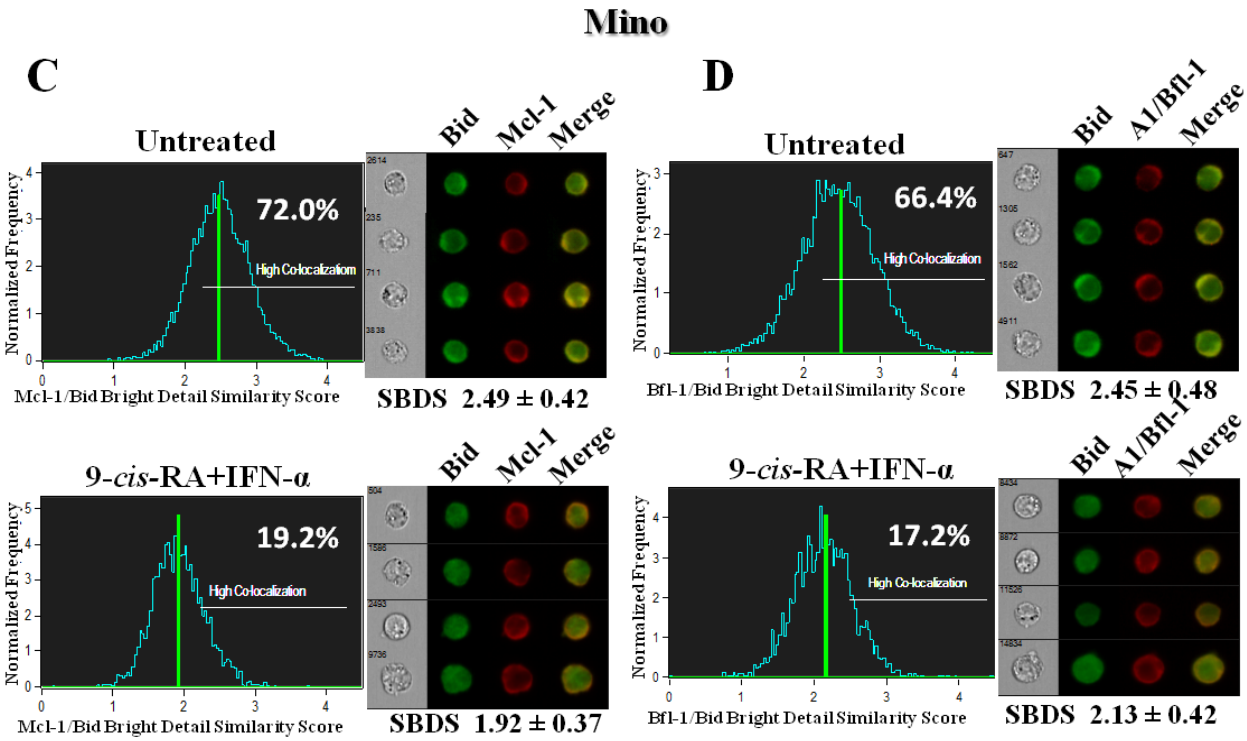
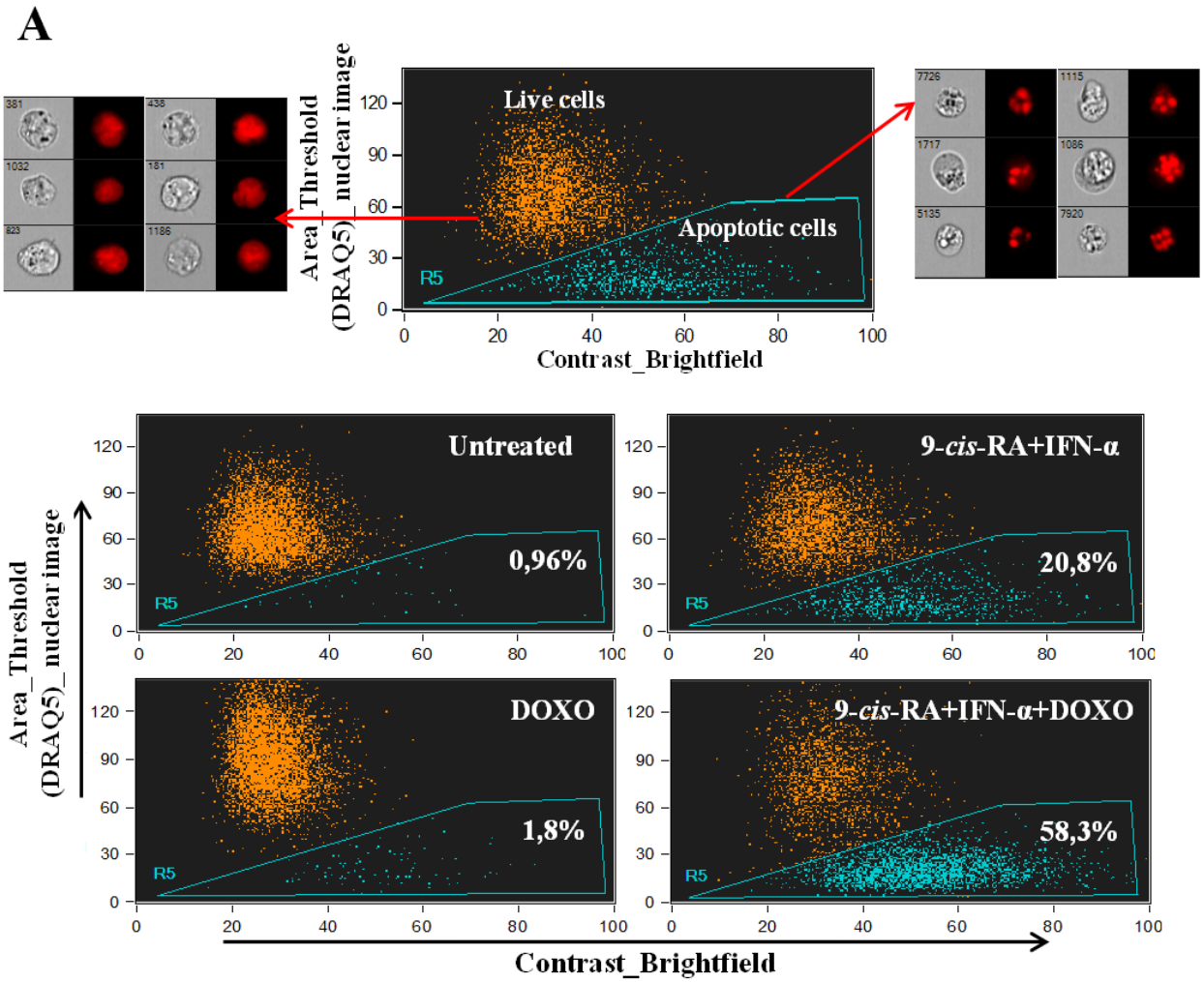


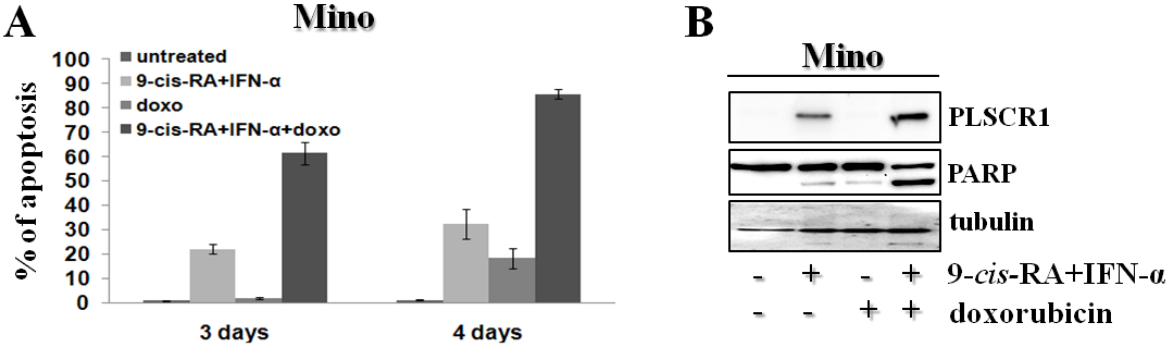
Figure 8. ***A)*** **9-*cis*-RA/IFN-α combination promotes the formation of Noxa/Mcl-1 and Noxa-A1/Bfl-1 complexes.** SP53 cells were cultured in absence or presence of 9-*cis*-RA/IFN-α for 3 days. Mcl-1 (left) and A1/Bfl-1 (right) were immunoprecipitated from 500 µg of total proteins. Immunoprecipitated (IP; bound) and non-immunoprecipitated (unbound) fractions were analyzed by immunoblotting for Noxa, Mcl-1, and A1/Bfl-1 proteins. ***B)*** **9-*cis*-RA/IFN-α-induced Noxa favors Bid** displacement from Bid-Mcl-1 complexes in SP53 cells (3 days of treatment). Mcl-1 was immunoprecipitated from 500 µg of total proteins followed by immunoblotting with antibodies against Noxa, full-length Bid, and Mcl-1. Data depicted in *A)* and *B)* are representative of three independent experiments. ***C)*** and ***D)*** **9-*cis*-RA/IFN-α treatment inhibits Bid-Mcl-1 and Bid-A1/Bfl-1 interactions *in vivo*.** Mino cells were cultured in absence (untreated) or presence of 9-*cis*-RA/IFN-α for 3 days, then 10^6 cells per sample were labeled with primary antibodies against Bid (1:50) and *C)* Mcl-1 (1:100) or *D)* A1/Bfl-1 (1:75). The vital nuclear dye DRAQ5 was added to each sample. $20x10^3$ cells were acquired with the ImageStream X and analyzed with a specific algorithm for the SBDS calculation. Data are representative of one of three independent experiments.

5. 9-*cis*-RA/IFN-α treatment sensitizes MCL cells to doxorubicin- and bortezomib-dependent apoptosis through PLSCR1 up-regulation.

Considering the pro-apoptotic effect of 9-*cis*-RA/IFN-α in MCL cells, we next investigated whether pre-treatment with this drug combination could synergistically increase the apoptotic effects of other drugs currently employed in MCL management, such as doxorubicin (DOXO) and bortezomib (BTZ). To this end, Mino cell line were cultured in the absence or presence of 9-*cis*-RA/IFN-α for 48 hours and then DOXO (50 nM) or BTZ (0,5 nM) were added for 24 and 48 hours. As shown in Figure 9A and 9C, incubation of 9-*cis*-RA/IFN-α pre-treated Mino cells with DOXO or with BTZ significantly enhanced the extent of apoptosis induced by either agent alone. In particular, the synergistic effect between 9-*cis*-RA/IFN-α combination and DOXO or BTZ was noted as early as 24 hours following DOXO and BTZ addition and it reached up to 85% of apoptosis after 48 hours (Figure 9A). Similar results were obtained for SP53 and Jeko-1 cell lines (not shown). Apoptosis was detected by multispectral imaging flow cytometry which allows distinguishing between viable and apoptotic cells on the basis of the nuclear morphology using a nuclear staining. A representative experiment (Figure 9A) showed how this analysis efficiently split the cells into two clearly distinguished populations: one accounts for viable cells with intact nucleus, and the other for apoptotic cells characterized by nuclear fragmentation. Detection of PARP and cleaved caspase 3 by immunoblotting confirmed the relative extents of apoptosis (Figure 9B and 9D). Intriguingly, immunoblotting analysis demonstrated that the enhanced apoptotic levels induced by the combination of the three drugs are associated with a further PLSCR1 up-regulation compared to 9-*cis*-RA/IFN-α alone, while no expression of the protein was detectable in cells treated with DOXO or BTZ alone (Figure 9B and 9D). The role of

PLSCR1 in the sensitization of MCL cells to DOXO- or BTZ-dependent apoptosis was further evaluated by taking advantage from PLSCR1-knocked down Mino cells. As shown in Figure 9E and 9F, PLSCR1 silencing significantly decreased the extent of apoptosis in cells pre-treated with 9-*cis*-RA/IFN-α and successively exposed to DOXO or BTZ, suggesting that 9-*cis*-RA/IFN-α-induced PLSCR1 expression plays a pivotal role in making MCL cells more responsive to DOXO and BTZ treatments.





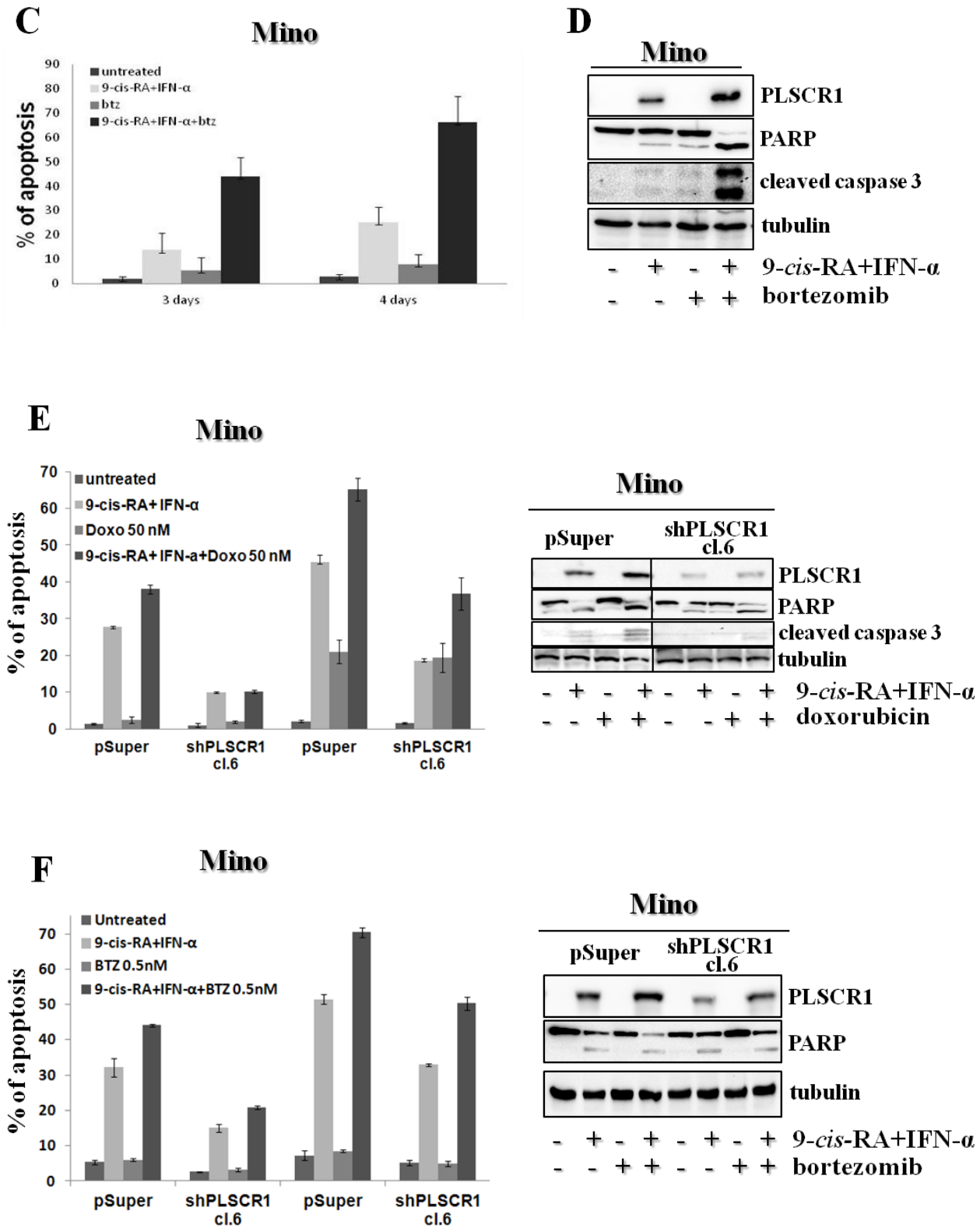


Figure 9. ***A)*** and ***B)*** **9-*cis*-RA/IFN-α treatment sensitizes MCL cells to *A)* doxorubicin- or *B)* bortezomib-dependent apoptosis**. Mino cells were sequentially treated with 9-*cis*-RA/IFN-α for 48 hours, then 50 nM DOXO or 0,5 nM BTZ was added for 24 and 48 hours. 10^6 cells per sample were labeled with the vital nuclear dye DRAQ5 and fixed. $20x10^3$ cells were acquired with ImageStreamX. Representative dot plots and cell images show how this

analysis distinguishes apoptotic cells on the basis of the nuclear fragmentation. Results from three independent experiments (mean ± SD). ***C)*** and ***D)*** **Doxorubicin *C)* and bortezomib *D)* enhances 9-*cis*-RA/IFN-α-dependent PLSCR1 up-regulation**. 50 μg of proteins were analyzed for PLSCR1, PARP, and cleaved caspase 3 detection. Tubulin analysis was included as a protein loading control. ***E)*** and ***F)*** **PLSCR1 is involved in 9-*cis*-RA/IFN-α/DOXO or BTZ synergic pro-apoptotic effects.** Mino cells infected with empty vector pSuper or vector containing shPLSCR1 (cl.6) were pre-treated with RA/IFN-α for 48 hours, and then were exposed to doxorubicin 50 nM or to bortezomib 0,5 nM for the next 24 and 48 hours. The percentage of apoptotic cells were detected by ImageStreamX technology. Bars, mean from three replicates; error bars, SD. The association between enhanced up-regulation of PLSCR1 and the increased apoptosis was confirmed by immunoblotting analysis, in the same samples, of the active form of caspase 3 and cleaved PARP. Tubulin analysis was included as a protein loading control.

6. 9-*cis*-RA/IFN-α-dependent PLSCR1 up-regulation does not enhance the pro-apoptotic effects of rituximab.

One of the most widely used drugs in the treatment of MCL is given by the anti-CD20 monoclonal antibody rituximab (RTX). In fact, the high expression levels of CD20 on the cell surface of MCL B-cells makes it an attractive target for RTX treatment [Lenz *et al*., 2005]. Nonetheless, RTX has usually a limited efficacy in this setting if used as a single agent, being thus more commonly used in combination with other chemotherapy drugs. On the basis of the results obtained, we therefore investigated the effects of a possible combination therapy including RTX and 9-*cis*-RA/IFN-α. Given the ability of 9-*cis*-RA/IFN-α-induced PLSCR1 to enhance MCL cells sensitivity to DOXO and BTZ treatments, we explored whether the presence of PLSCR1 could affect also RTX antitumor activities in MCL. To this end, Mino, SP53 and Jeko-1 cell lines were pre-treated with 9-*cis*-RA/IFN-α for 48 hours to induce PLSCR1 expression, then these cells were exposed to RTX (10 μg ml-1) for further 24 or 48 hours, and analyzed for apoptosis. Data obtained demonstrated that pre-treatment with 9-*cis*-RA/IFN-α does not sensitize MCL cells to RTX-dependent apoptosis (Figure 10A). Since one of the main effects of RTX is to promote natural killer (NK)-mediated antibody dependent cytotoxicity, we evaluated the ability of NK cells to kill MCL cells labelled with RTX in absence or presence of 9-*cis*-RA/IFN-α. The results showed that the treatment does not enhance the recognition of MCL cells by NK cells and does not influence ADCC (not shown). Moreover, the addition of RTX to the 9-*cis*-RA/IFN-α combination did not induce synergic effects on PLSCR1 induction (Figure 10A). The absence of any additive/synergic effect between these drugs could be explained by the fact that 9-*cis*-RA/IFN-α pre-treatment markedly reduces the expression of CD20 on the membrane surface of MCL cells, thus interfering with RTX mechanism of action. In fact, flow cytometric analysis of CD20 expression showed that it decreases as early as 24 hours following

drugs exposure, though the effect is greater after 48 hours of treatment in all the three MCL cell lines analyzed (Figure 10B). These data are clinically relevant as they suggest that the employment of RTX treatment in combination with 9-*cis*-RA/IFN-α is not advisable in the practice.

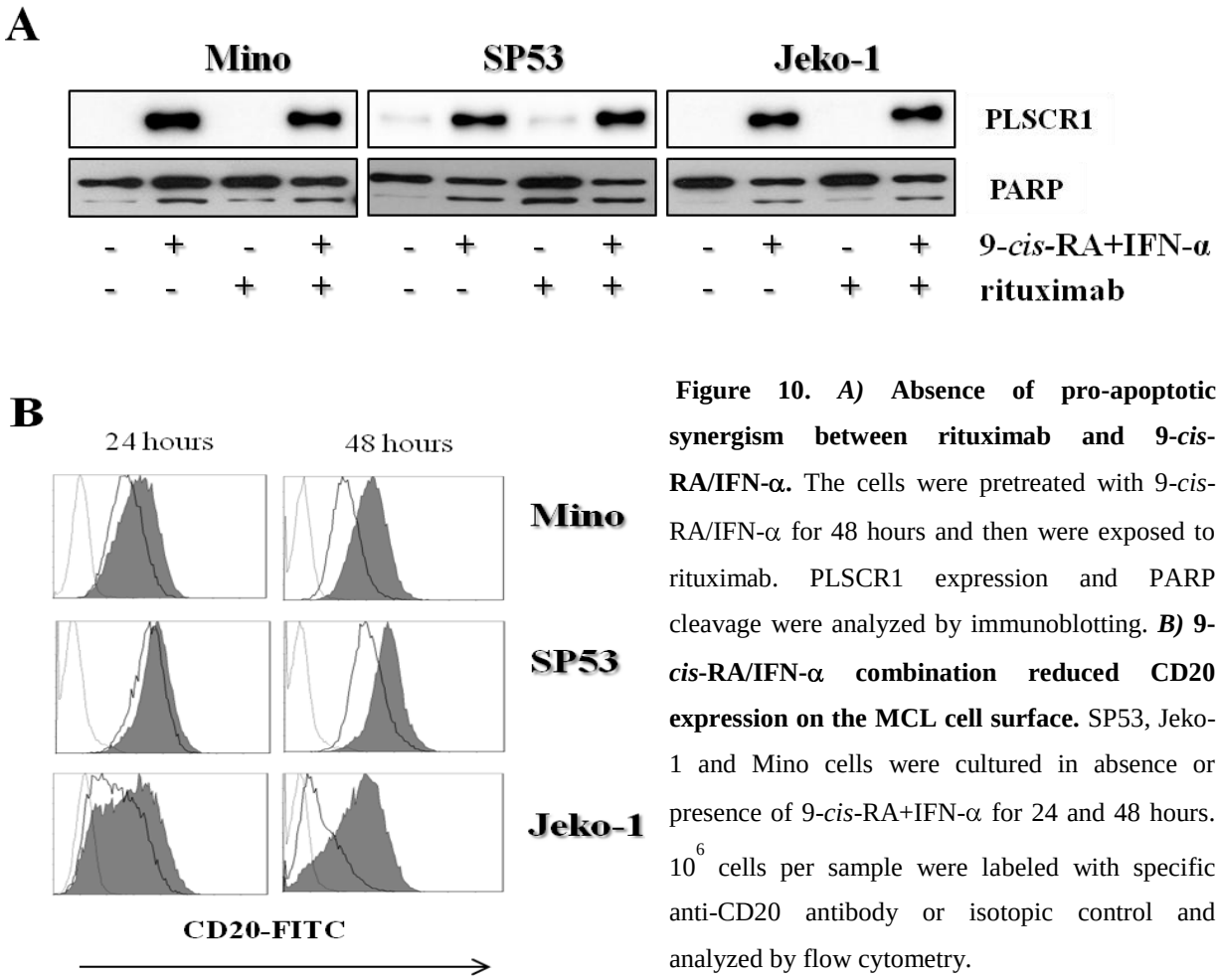


Figure 10. ***A)*** **Absence of pro-apoptotic synergism between rituximab and 9-*cis*-RA/IFN-α.** The cells were pretreated with 9-*cis*-RA/IFN-α for 48 hours and then were exposed to rituximab. PLSCR1 expression and PARP cleavage were analyzed by immunoblotting. ***B)*** **9-*cis*-RA/IFN-α combination reduced CD20 expression on the MCL cell surface.** SP53, Jeko-1 and Mino cells were cultured in absence or presence of 9-*cis*-RA+IFN-α for 24 and 48 hours. 10^6 cells per sample were labeled with specific anti-CD20 antibody or isotopic control and analyzed by flow cytometry.

7. PLSCR1 is heterogeneously expressed in tumor biopsies of MCL.

Given the pro-apoptotic role of PLSCR1 in MCL and the variability of its basal expression detected in the cell lines included in this study, we analyzed by immunohistochemistry and immunoblotting, respectively, 28 biopsies and 4 MCL primary cultures for the presence of this protein. Considering positive only the cases with more than 10% cells expressing PLSCR1 protein, we identified 9 samples in which this percentage fluctuates between 11% and 40%, and evaluated the association between PLSCR1 expression and the proliferation index Ki-67 using the two-tailed independent t test. Preliminary data indicated that the cell proliferation is significantly higher in PLSCR1 positive samples than in negative ones ($P < 0.005$) (Figure 11A). Furthermore, immunoblotting analysis of the primary cultures obtained from MCL samples with

no detectable levels of PLSCR1 and exposed to 9-*cis*-RA/IFN-α for 48 hours, confirmed a significant treatment-dependent PLSCR1 up-regulation in all 4 samples (MCL4, MCL5, MCL6, MCL7) (Figure 11B). Interestingly, as happened for MCL cell lines, the PLSCR1 induction correlated with a pro-apoptotic effect shown by the increased expression of cleaved caspase 3 (Figure 11B). A larger scale study may allow to define whether PLSCR1 could be a new biomarker for MCL classification and to evaluate its potential association with clinical-pathological parameters.

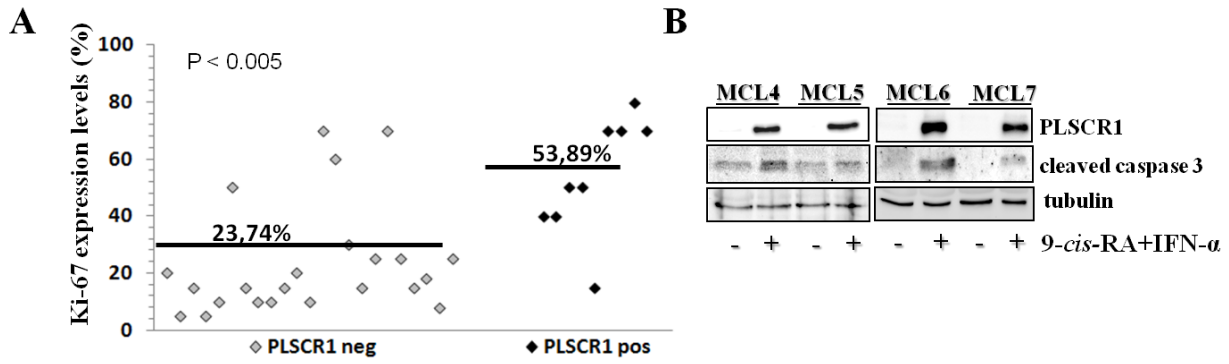


Figure 11. ***A)*** **Correlation between PLSCR1 expression and Ki-67.** Expression levels of the proliferation index Ki-67 in PLSCR1 negative and positive (threshold: 10%) MCLs. Two-tailed independent *t* test. ***B)*** **RA/IFN-α combination induces PLSCR1 up-regulation associated with caspase 3 activation in primary MCL cultures.** Purified primary lymphoma cells (MCL4, MCL5, MCL6, and MCL7) were treated for 48 hours. Total-cell lysates (30 µg) were subjected to immunoblotting analysis for the detection of PLSCR1. The association between PLSCR1 induction and RA/IFN-α-dependent apoptosis was confirmed by immunoblotting analysis of the active/cleaved form of caspase 3. Tubulin analysis is included as a protein loading control.

8. STAT1 is the mediator of 9-*cis*-RA/IFN-α-promoted PLSCR1 transcription in MCL cells.

The signal transducer and activator of transcription 1 (STAT1) is an essential transcription factor for the expression of the majority of IFN-induced genes [Durbin *et al.*, 1996], including PLSCR1 [Zhao *et al.*, 2005]. In response to IFNs, indeed, STAT1 is phosphorylated at tyrosine 701 (Y701) and this post-translational modification is necessary for STAT1 dimerization, nuclear translocation and DNA binding [Decker *et al.*, 1997]. In addition, Y701 phosphorylation is needed for the subsequent phosphorylation at serine 727 (S727) that causes the protein to acquire its full transcriptional activity [Wen *et al.*, 1995; Kovarik *et al.*, 2001]. Immunoblotting analysis showed that 9-*cis*-RA/IFN-α rapidly and time-dependently results in an increased STAT1 phosphorylation at both Y701 and S727 in SP53 and Jeko-1 cell lines (Figure 12A).

Moreover, treatment-induced STAT1 activation is a very early event, detectable just 15 minutes after drugs exposure (Figure 12A). Taking advantage from ImageStreamX technology, we evaluated the translocation of STAT1 into the nucleus after 9-*cis*-RA/IFN-α stimulation. SP53 and Jeko-1 cells were harvested at different time points of drugs exposure, permeabilized and stained with the DRAQ5 nuclear dye and a specific anti-phospho-STAT1 Y701 antibody. Nuclear localization of phospho-STAT1 was analyzed gating only positive cells and was measured on a per-cell basis using the Similarity Score (SS) index (see materials and methods). Representative histograms of data recorded at 3 time points are shown in Figure 12B. Phospho-STAT1 Y701 was completely localized in the cytoplasm in cells with a low SS, while the intracellular distribution of this transcription factor changes accordingly with a nuclear internalization in cells with a high SS. Overall, this analysis demonstrated that the percentage of cells showing a nuclear localization of phospho-STAT1 Y701 is significantly increased in both cell lines just after 15 minutes of drugs exposure, it remains quite constant for 2 hours and then starts to decrease from 74% to 23% in SP53, and from 78% to 65% in Jeko-1 cells. Moreover, using a noshift transcription factor assay for different proteins, such as STAT1, ATF2, c-Myc, MEF-2 and c-Jun, we demonstrated that 9-*cis*-RA/IFN-α treatment specifically affects STAT1 activity, while leaves unchanged the other factors analyzed (Figure 12C). In particular, STAT1 activity increases up to 4.1 fold in SP53 and up to 8.7 fold in Jeko-1 cells following 4 hours of treatment. These results indicate that the treatment induces a functional STAT1 activation, suggesting a role for this transcription factor in mediating 9-*cis*-RA/IFN-α-dependent PLSCR1 up-regulation.

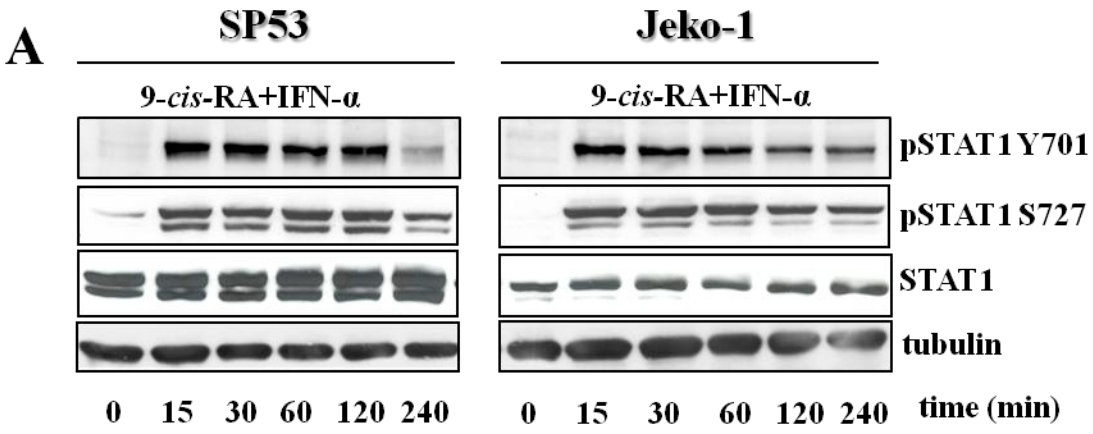
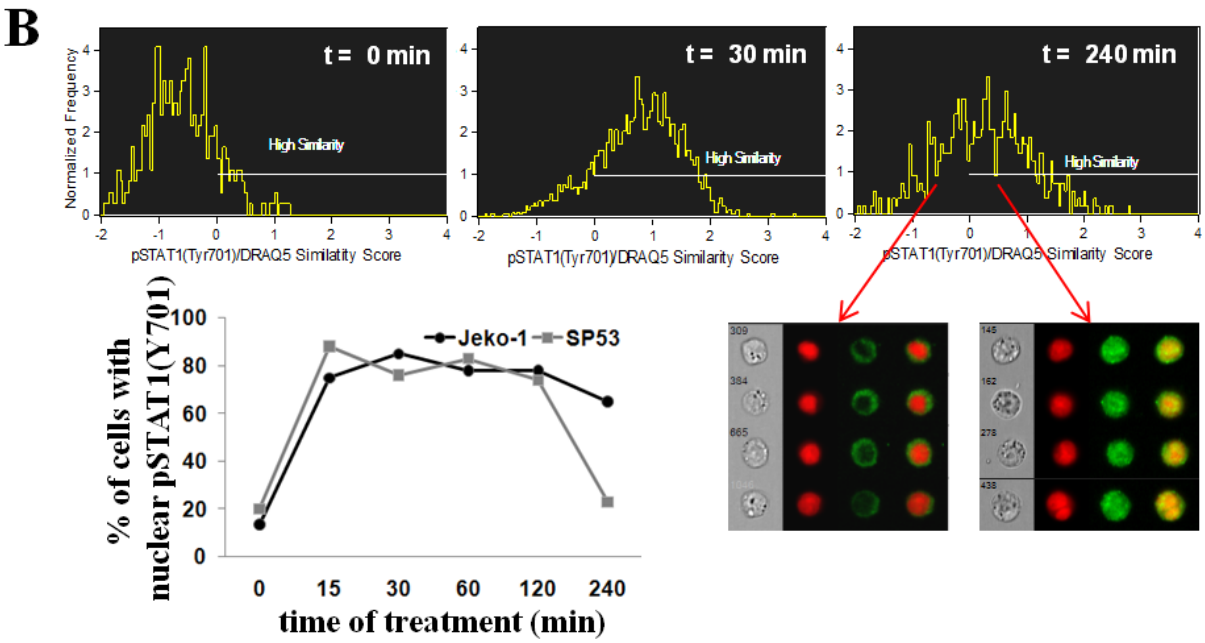
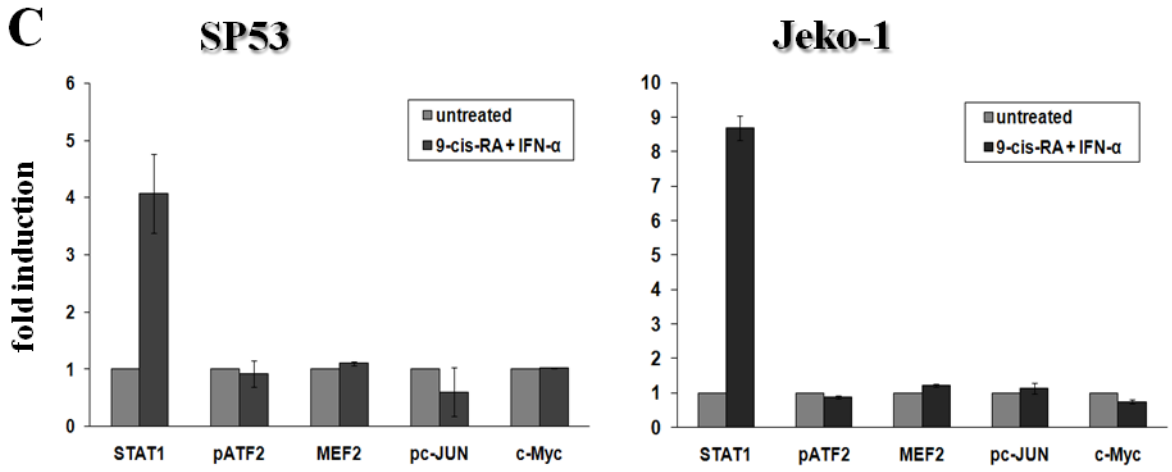


Figure 12. ***A)*** **9-*cis*-RA/IFN-α treatment induces STAT1 activation.** SP53 and Jeko-1 cells were treated with 9-cis-RA/IFN-α combination and the phosphorylation status of STAT1 was evaluated at the indicated time points. Tubulin shows equal loading of proteins for each lane. ***B)*** 9-*cis*-RA/IFN-α-dependent STAT1 phosphorylation is associated with its nuclear internalization. 9-*cis*-RA/IFN-α treated SP53 and Jeko-1 cells were harvested at the

indicated time points. 20x10^3 cells were acquired with ImageStream X and phospho-STAT1 nuclear localization was calculated as Similarity Score between phospho-STAT1 and DRAQ5 intensities. All events showing a positive SS were considered with high similarity between phospho-STAT1 and DRAQ5, thus indicating a nuclear localization of phospho-STAT1. Data are representative of one of three independent experiments. ***C)* 9-*cis*-RA/IFN-α treatment specifically promotes STAT1 activity.** The cells were treated or not with 9-*cis*-RA/IFN-α (4 hours). 15 µg of nuclear protein extracts were analyzed for the DNA-binding activity of the indicated transcription factors using TransAM MAPK family transcription factors assay.





9. STAT1-mediated PLSCR1 up-regulation in MCL is antagonized by Akt pharmacological inhibition.

Among the pathways involved in the tumorigenesis of MCL, the constitutively activated PI3-K/Akt pathway was shown, by us and others, to have a crucial role in regulating both cell proliferation and survival [Peponi *et al*., 2006, Dal Col *et al*., 2008]. It has been reported that inhibition of PI3-K abolishes IFNγ-induced phosphorylation of STAT1 S727 and reduces STAT1-driven transcription in human fibrosarcoma and human glioblastoma cell lines [Nguyen *et al*., 2001]. On these grounds, a time course analysis of Akt activation in SP53 and Jeko-1 cells treated with 9-*cis*-RA/IFN-α revealed that a short-time exposure, between 2 and 4 hours, to the treatment enhances Akt phosphorylation, which decreases after 24 hours of treatment (Figure 13A). Therefore, the possible involvement of Akt in 9-*cis*-RA/IFN-α-induced STAT1 activation and PLSCR1 up-regulation was evaluated in SP53 and Jeko-1 cell lines after 1 hour of pre-treatment with the Akt specific inhibitor SH5 (10 μM), followed by 4 and 24 hours exposure to 9-*cis*-RA/IFN-α. Interestingly, Akt pharmacological inhibition significantly contrasts the effect of 9-*cis*-RA/IFN-α on PLSCR1 induction via STAT1 inhibition (Figure 13B). These results demonstrate that 9-*cis*-RA/IFN-α combination has a time-dependent effect on Akt activation in MCL cells, inducing an early increase of its activating phosphorylation followed by a strong inhibition of the kinase activity after the first 24 hours of exposure.

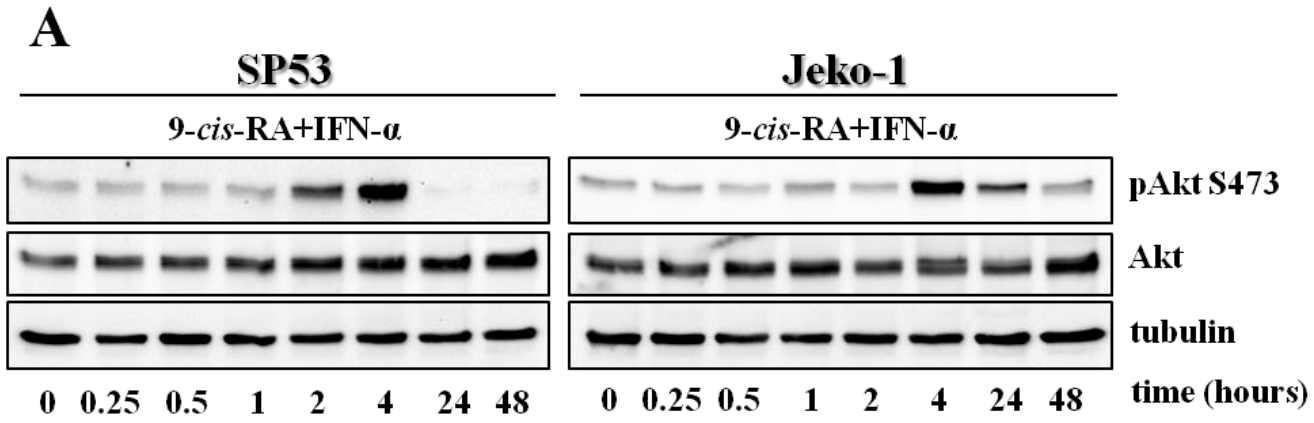
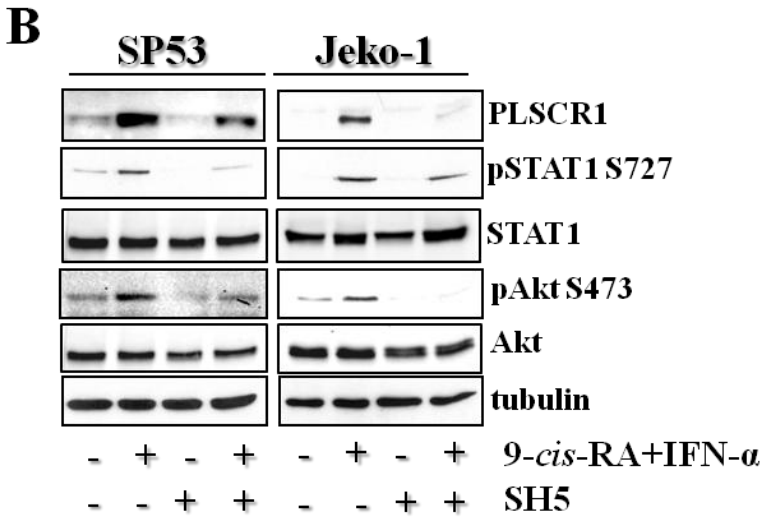


Figure 13. *A*) Short exposure to 9-*cis*-RA/IFN-α enhances Akt activation in MCL cells. Phosphorylation status of Akt in SP53 and Jeko-1 cells treated with 9-*cis*-RA/IFN-α. ***B*) 9-*cis*-RA/IFN-α-induced Akt activation is involved in treatment-dependent PLSCR1 up-regulation.** SP53 and Jeko-1 cells were pre-treated with SH5 10 μM for 1 hour and then were exposed to 9-*cis*-RA/IFN-α. Phospho-protein expression was analyzed after 4 hours of treatment, while protein extracts from 24 hours treated cells were used for PLSCR1 immunoblotting analysis. Tubulin shows equal loading of proteins for each lane.

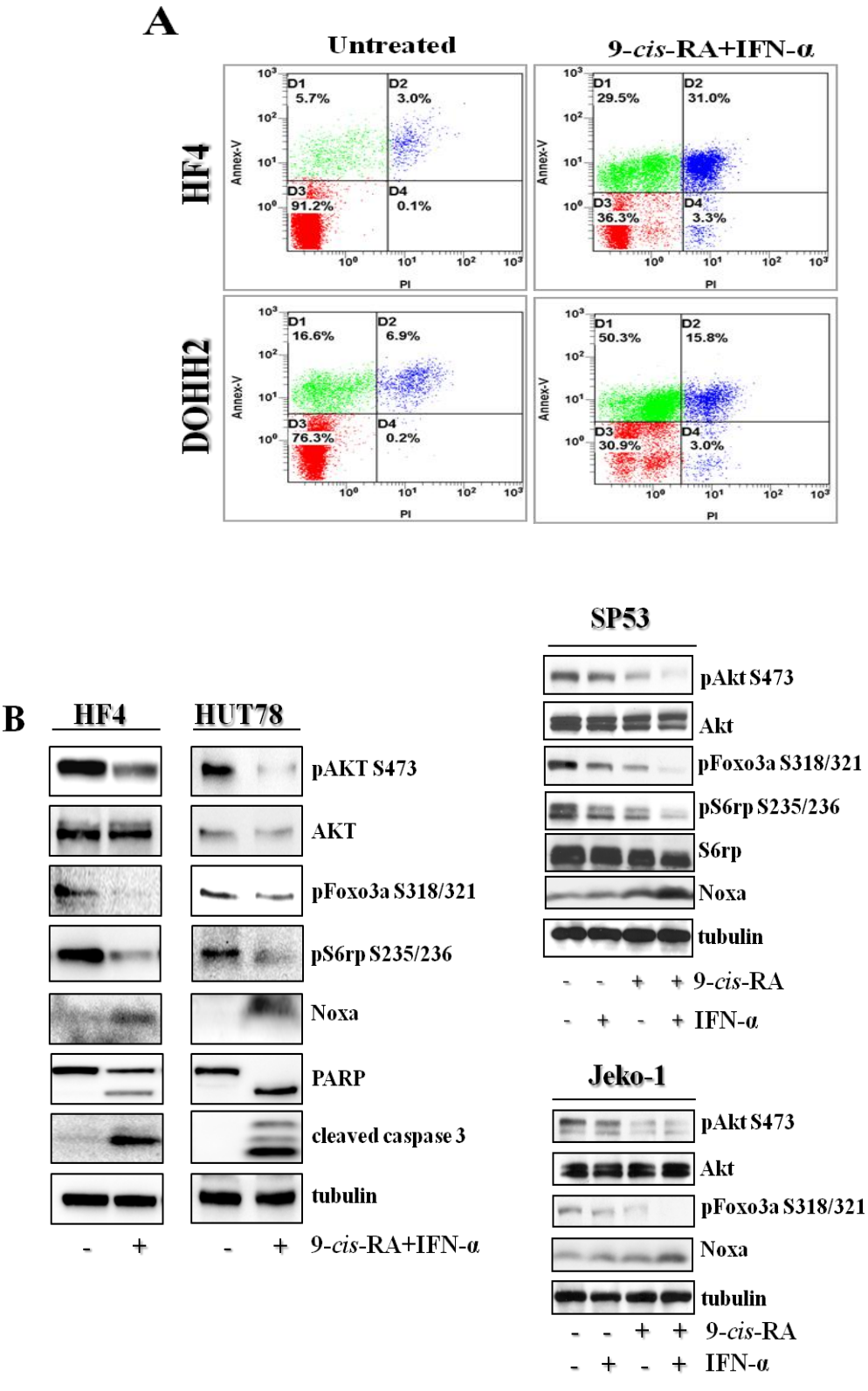


10. 9-*cis*-RA/IFN-α combination induces significant pro-apoptotic effects in various lymphoma histotypes characterized by inherent Akt activation.

Constitutive activation of the PI3-K/Akt pathway is a critical deregulation of neoplastic cell signaling, and tumor cells carrying an inherently activated Akt may depend on this kinase for proliferation and resistance to apoptosis. Activation of the PI3-K/Akt/mTOR pathway, for instance, is required for FL growth, as rapamycin inhibits the growth of FL cell lines *in vitro*, and in xenograft models [Bhende *et al*. 2010]. Moreover, not only MCL cells but also spontaneously growing CTCL-derived cell lines show, among the other molecular alterations, a persistent activation of this pathway [Marzec *et al*., 2008]. Interestingly, NVP-BEZ235, a dual PI3-K and mTOR inhibitor, is effective in inhibiting FL proliferation and increases FL apoptotic response [Bhende *et al*. 2010] and we recently demonstrated that pharmacological inhibition of constitutively active PI3-K or Akt induced significant levels of apoptosis in both established cell lines and primary MCL cultures [Dal Col *et al.,* 2008]. The anti-proliferative and pro-apoptotic effects of 9-*cis*-RA/IFN-α on MCL demonstrated so far, together with the known activity of these drugs in the clinical practice for the treatment of CTCL, suggest that this combination may have similar effects in other hematologic malignancies such as FL. On these grounds, we investigated the ability of 9-*cis*-RA/IFN-α to trigger apoptotic effects also in CTCL and FL cell lines and, in particular, if this response may occur through a down-regulation of the PI3-K/Akt/mTOR pathway. Therefore, DOHH2, HF4, HUT78, SP53 and Jeko-1 cell lines were treated with 9-*cis*-RA/IFN-α for 3 days and analyzed for apoptosis and for the presence of the phosphorylated form of Akt and of its substrate FOXO3a. In addition, TORC1 activation was investigated by studying the phosphorylation of one of its main substrates, the S6 ribosomal protein. Notably, 9-*cis*-RA/IFN-α combination induced apoptosis also in CTCL and FL cell

lines, as shown by the percentage of AnnexinV/PI positive cells and by the presence of PARP and caspase 3 cleaved forms (Figure 14A and 14B), and inhibited Akt and mTOR activation in all cell lines analyzed, as shown by the down-regulation of phospho-(S473)-Akt, of its substrate phospho-(S318/321)-FOXO3a, and of phospho-(S235/236)-S6RP, respectively (Figure 14B). More importantly, 9-*cis*-RA/IFN-α-induced Akt inhibition was associated with Noxa up-regulation (Figure 14B) in all the different types of lymphoma cells studied. In addition, this event was observed in MCL cell lines carrying either wild-type (SP53) or mutant (Jeko-1) p53 (Figure 14B), supporting the hypothesis that a different transcription factor from p53 is involved in this phenomenon. Noteworthy, FOXO3a activates the transcription of several genes, including PMAIP1, which encodes for the Noxa protein [Obexer *et al*. 2007]. FOXO3a transcriptional activity is regulated by the control of its intracellular localization through the phosphorylation/dephosphorylation of different serine/threonine residues. In particular, Akt-dependent phosphorylation on Thr32, Ser318/321, and Ser253 abolishes its nuclear translocation [Tizivion *et al*., 2011]. Given the ability of 9-*cis*-RA/IFN-α to inhibit Akt-dependent FOXO3a phosphorylation, using multispectral imaging flow cytometry we evaluated FOXO3a intracellular localization after 48 hours exposure to 9-cis-RA/IFN-α, SH5 (10 μM), or rapamycin (0.1 μM) in Mino cell line. As shown in Figure 14C, FOXO3a protein is clearly retained in the cytoplasm of untreated cells, whereas in 9-*cis*-RA/IFN-α- and SH5-treated cells, the protein is also detectable within the nucleus in 58.7% and 64.1% of cells, respectively. In contrast, rapamycin did not affect FOXO3a intracellular localization, suggesting that this transcription factor is Akt- but not TORC1-dependent (Figure 14C). Notably, the analysis were performed excluding apoptotic cells, given that FOXO3a nuclear internalization and consequent Noxa up-regulation are two events occurring in the first steps of the apoptotic process. These results are consistent with a role of FOXO3a as a molecular mediator of the 9-*cis*-RA/IFN-α-induced Noxa up-regulation. Moreover, taking into account our previous findings indicating that Akt but not TORC1 kinase is critical for MCL cell survival [Dal Col *et al.*, 2008], we also investigated if mTOR inhibition could induce apoptotic response in FL cell lines. Surprisingly, 48 hours treatment of DOHH2 and HF4 cells with the Akt-specific inhibitor SH5, but not with the TORC1-specific inhibitor rapamycin, induced caspase 3 activation (Figure 14D) and thus apoptosis (Figure 14E), suggesting that the general mechanism of 9-*cis*-RA/IFN-α-induced cell death for different type of non-Hodgkin lymphoid malignancies depends on Akt but not on mTOR inhibition. Furthermore, consistently with our hypothesis and previous findings, the PI3-K/Akt inhibitor LY294002 (50 μM) induced a marked up-regulation of the apoptotic protein Noxa and a complete depletion of the anti-apoptotic protein A1/Bfl-1 in the SP53 cell line,

whereas rapamycin did not affect the levels of these proteins (Figure 14F). Overall, our data provide evidence that the molecular mechanism underlying 9-*cis*-RA/IFN-α-dependent apoptosis of Akt-driven NHLs involves FOXO3a dephosphorylation/activation and its subsequent nuclear internalization followed by Noxa up-regulation as a consequence of Akt inhibition (Figure 15A and 15B).



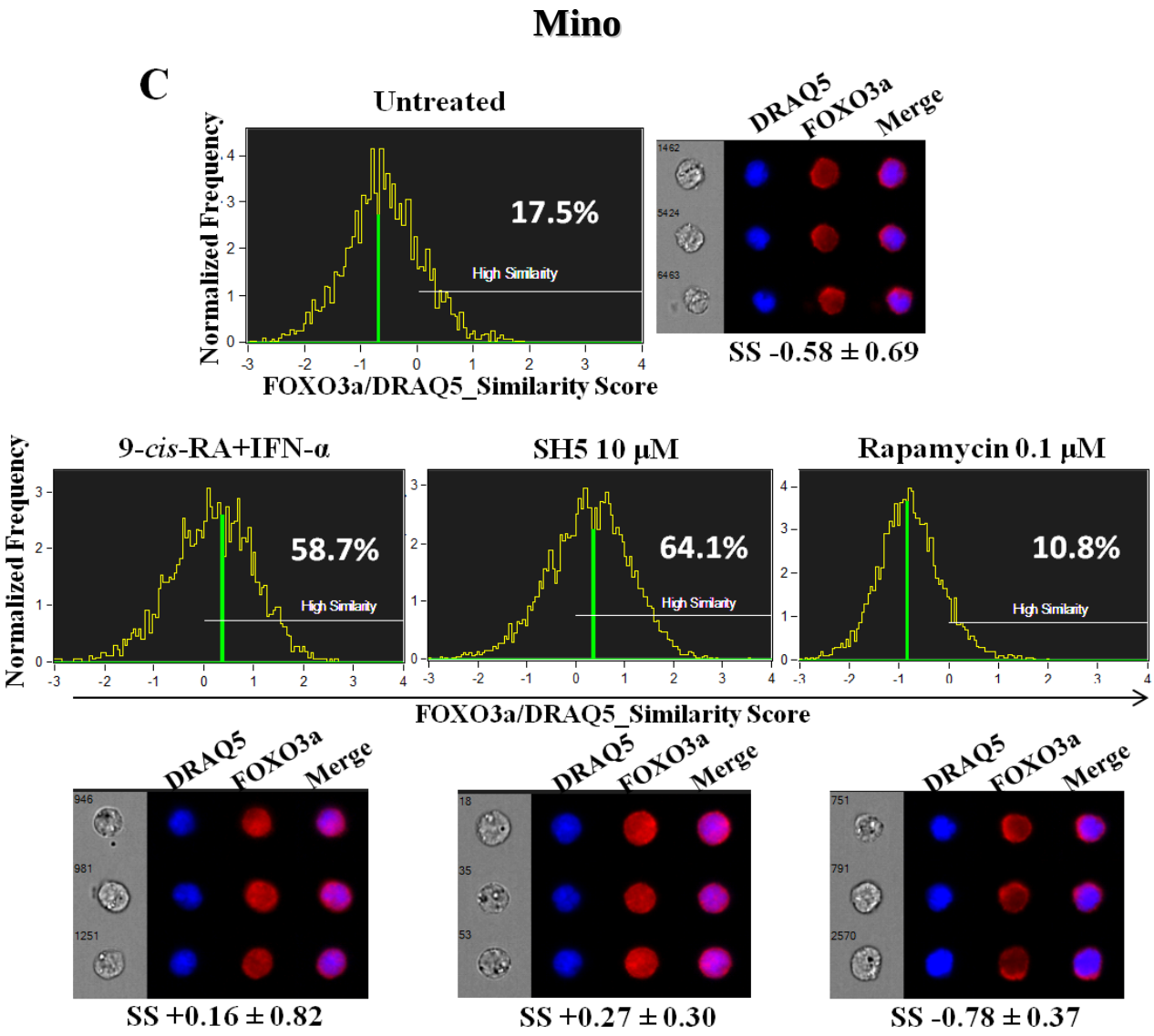
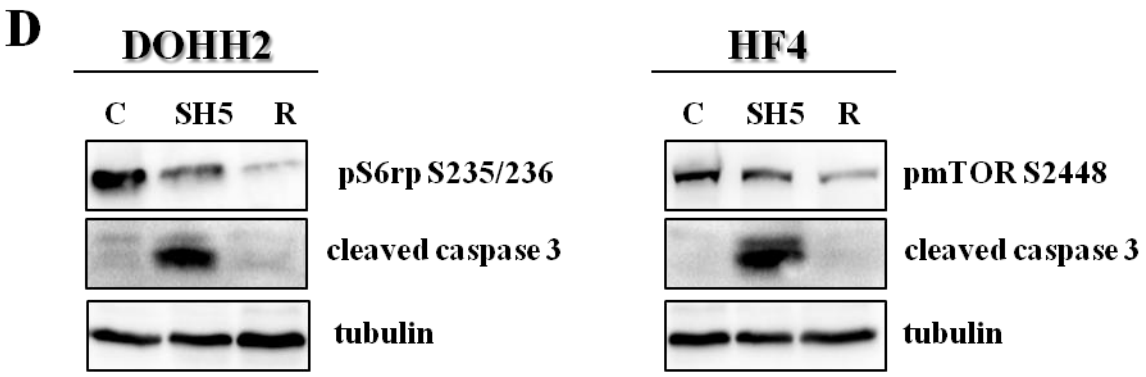


Figure 14. ***A)*** and ***B)*** **9-*cis*-RA/IFN-α combination induces apoptosis in FL and CTCL cell lines.** DOHH2, HF4, and HUT78 cell lines were treated with 9-*cis*-RA/IFN-α. Apoptosis was evaluated after 3 days with AnnexinV/PI (*A*) or with immunoblotting analysis of the active form of caspase 3 and cleaved PARP (*B*). ***B)*** **9-*cis*-RA/IFN-α combination inhibits the inherent PI3-K/Akt pathway activation in SP53, Jeko-1, HF4, and HUT78 cells** (72 hours of treatment). Total-cell lysates, (100 μg) were subjected to immunoblotting using phospho-specific and anti-Noxa antibodies. ***C)*** **9-*cis*-RA/IFN-α treatment promotes FOXO3a nuclear localization.** Mino cells were untreated or treated with 9-*cis*-RA/IFN-α, SH5 (10 μM), or rapamycin (0.1 μM) for 48 hours and labeled with antibody against FOXO3a (1:100) and the vital nuclear dye DRAQ5. Cells ($20x10^3$) were acquired with ImageStream X and analyzed with the IDEAS software. FOXO3a nuclear localization was calculated as Similarity Score between FOXO3a and DRAQ5 intensities. Data are representative of one of three independent experiments. ***D)*** and ***E)*** **Inhibition of Akt, but not of TORC1, is associated with apoptosis in DOHH2 and HF4 cell lines.** Cells were treated with SH5 (10 μM), or rapamycin (0.1 μM) for 48 hours and analyzed by immunoblotting with phospho-specific antibodies and for cleaved caspase 3 (*D*). Apoptosis analysis was conducted also by ImageStream Technology in DOHH2 treated with rapamycin (0.1 μM) (*E*). ***F)*** **Inhibition of Akt, but not of TORC1, is associated with Noxa up-regulation and A1/Bfl-1 depletion.** SP53 cells were untreated or treated with 50 μM LY294002 (LY) or 0.1 μM rapamycin (R) for 48 hours and total-cell lysates analyzed for the expression of phospho-Akt, Noxa, and A1/Bfl-1 proteins.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DOHH2
C
SH5
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pmTOR S2448
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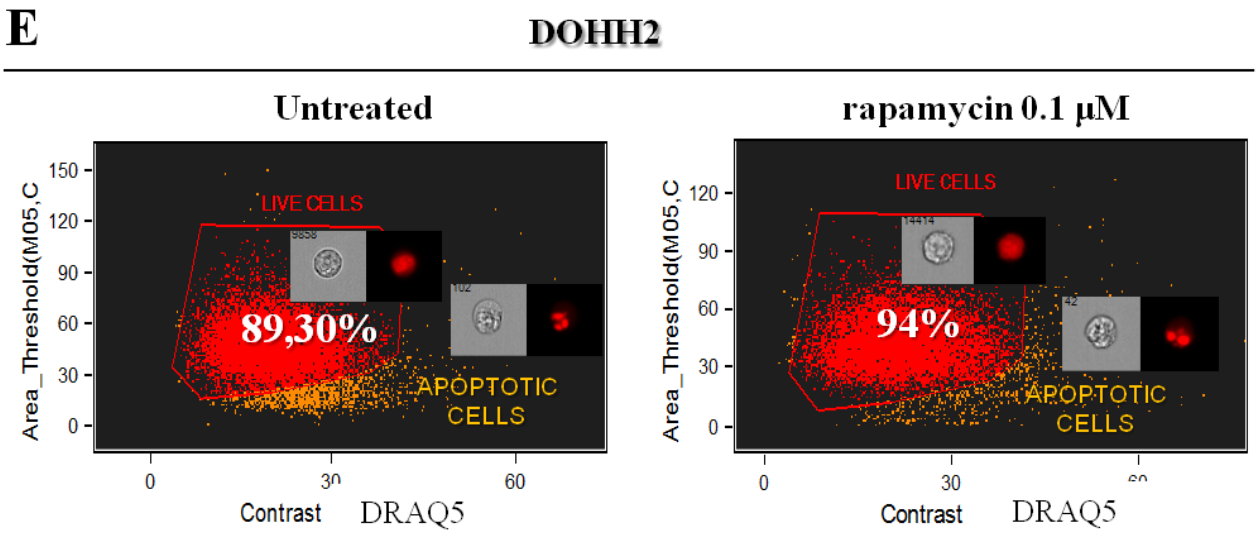
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DOHH2
Untreated
rapamycin 0.1 µM
LIVE CELLS
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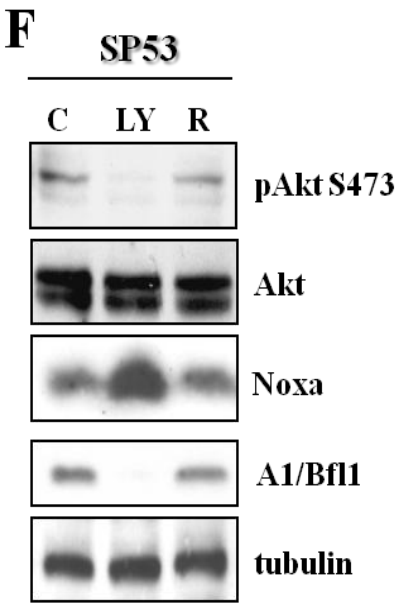
F
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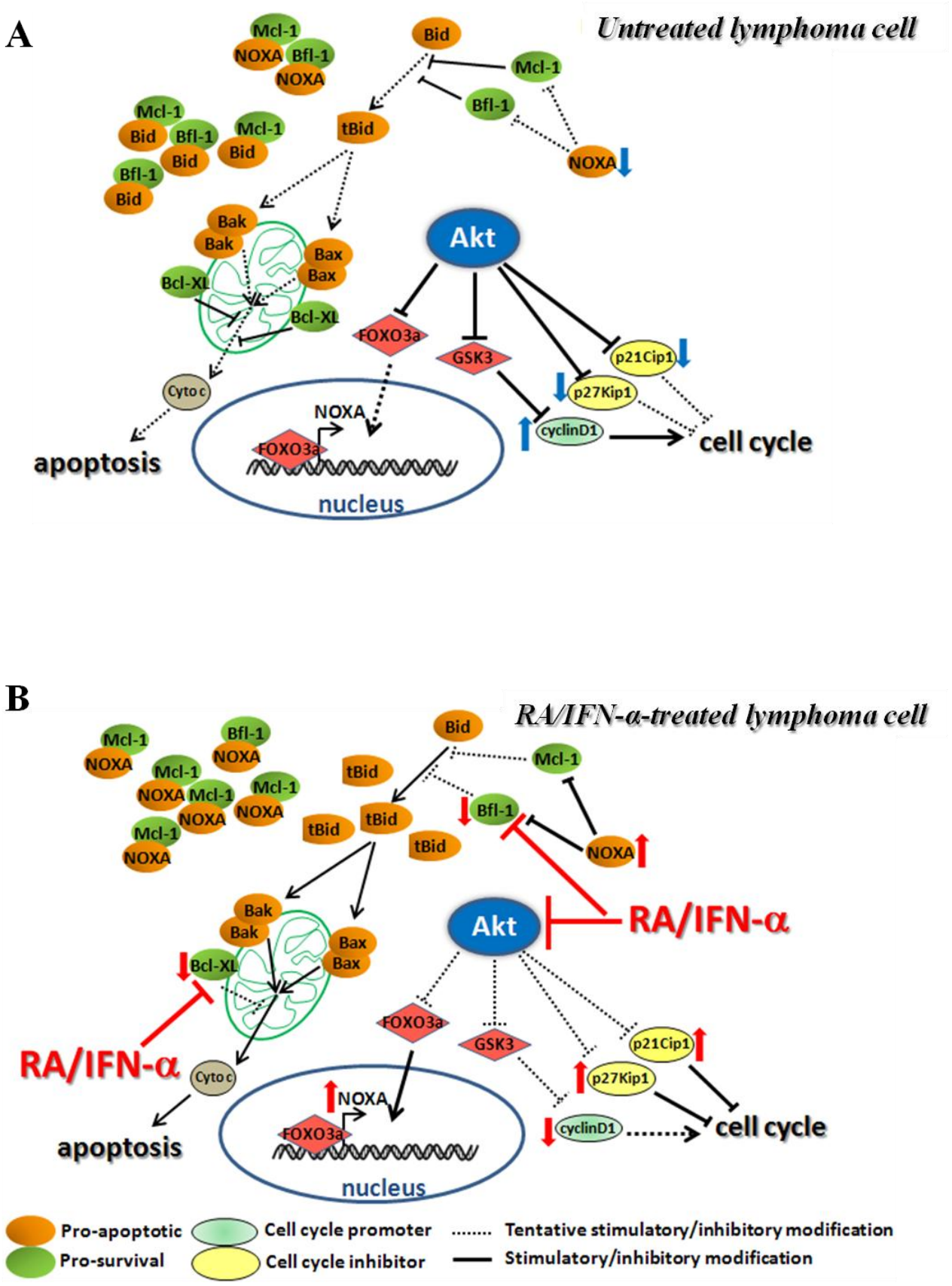

Figure 15. ***A)*** and ***B)*** Anti-proliferative and pro-apoptotic effects induced by 9-*cis*-RA/IFN-α in MCL cells. *A)* untreated lymphoma cells; *B)* modulation of critical regulators of lymphoma cells by 9-*cis*-RA/IFN-α.

DISCUSSION

As the understanding of the biology of tumors advances, novel agents rationally designed to target the key pathogenic mechanisms of cancers, such as cell cycle regulators and apoptotic proteins, continue to emerge. Previously, we demonstrated that the constitutive PI3-K/Akt/mTOR activation contributes to the stability of cyclin D1 and $p27^{Kip1}$ in MCL cells [Dal Col *et al*., 2008; Dal Col *et al*., 2008], suggesting that this signaling pathway may be a crucial therapeutic target in this and other lymphoma types where the pathway is deregulated, such as FL and CTCL [Marzec *et al*., 2008; Bhende *et al*., 2010]. In recent years, one of the major developments in lymphoma treatment has been, in fact, the perspective of the possible use of drugs targeting the oncogenic PI3-K/Akt pathway and its downstream effector mTOR. While the therapeutic potential of TORC1 inhibitors is being extensively studied in patients with relapsed or refractory MCL [Coiffier *et al*., 2009], specific inhibitors of the upstream kinase Akt are still under evaluation in phase I clinical trials, although these drugs are associated with a significant load of unwanted side effects [Perez-Galan *et al*., 2011]. However, the frequent emergence of resistance challenges the possible clinical use of classic mTOR inhibitors and is not yet clear why only a fraction of MCL patients respond and why the responses are not durable. Moreover, since Akt inhibition not only reduces proliferation, but also induces significant apoptotic responses [Dal Col *et al*., 2008], the Akt kinase may constitute a more effective target in MCL as compared to TORC1. Different PI3-K and Akt specific inhibitors, such as LY294002 and perifosine, have been already tested *in vitro* with lymphoma cell lines, and were shown to induce apoptosis [Chiarini *et al*., 2008]. Nevertheless, since this pathway is critical for a number of physiological functions, their use *in vivo* poses a significant concern regarding toxicity.

Herein, we demonstrate that RA/IFN-α co-treatment has significant effects on both proliferation and cell survival of lymphoma cells by affecting Akt constitutive activation. In particular, IFN-α enhances the anti-proliferative activity exerted by 9-*cis*-RA by inducing a down-regulation of cyclin D1, which is strongly over-expressed in most MCLs. Nevertheless, the observation that cyclin D1 over-expression alone is not sufficient for MCL development and that its down-regulation has only limited effects on MCL cell proliferation and survival [Klier *et al*., 2008] indicates that additional targets should be affected in order to obtain clinically relevant therapeutic efficacy. Intriguingly, the anti-proliferative activity of RA/IFN-α involves also the increased expression of the $p27^{Kip1}$ and $p21^{WAF1/Cip1}$ cell cycle inhibitors as a consequence of enhanced protein stability. This is particularly relevant for the $p27^{Kip1}$ protein, which shows an abnormally short half-life in most of MCLs [Chiarle *et al*., 2000]. Furthermore, $p21^{WAF1/Cip1}$ up-

regulation is induced irrespective of the p53 mutational status of the cells, thus excluding a p53-only-dependent effect and suggesting that this drug combination could be efficient also in cases showing deregulations in this critical pathway. This is particularly intriguing in the light of the observation that ≈25% of MCLs shows a deregulated p53 [Greiner *et al*., 2006; Stefancikova *et al*., 2010], a characteristic that could promote the resistance to novel drugs targeting the MDM2/p53 pathway, such as the MDM2 antagonist Nutlin-3 [Tabe *et al*., 2009] and MI-63 [Jones *et al*., 2008]. These findings are consistent with a relevant role of RA/IFN-α-induced Akt down-regulation in mediating MCL cell growth inhibition, as this drug combination mimics the effects induced in MCL cells by pharmacologic inhibition of Akt, which exactly results in cyclin D1 down-regulation and $p27^{Kip1}$ overexpression [Dal Col *et al*., 2008; Dal Col *et al*., 2008]. More relevant in a therapeutic perspective, is the demonstration that, unlike RA alone [Guidoboni *et al*., 2005], the RA/IFN-α combination induces significant levels of apoptosis in both established cell lines and primary MCL cultures, as well as in FL and CTCL cell lines. The exposure of MCL cells to 9-*cis*-RA for 24 hours and the following addition of IFN-α indicate this sequential treatment as the most effective combination, providing thus the rationale for the design of appropriate treatment schedules. The RA/IFN-α combination triggers both the death receptor/extrinsic and the mitochondrial/intrinsic apoptotic pathways and promotes the activation of the pro-apoptotic effectors Bak and Bax. Moreover, RA/IFN-α treatment induced up-regulation of Noxa and the concomitant Mcl-1 and A1/Bfl-1 inactivation as a result of protein-protein interactions in MCL cells. Interestingly, 9-*cis*-RA/IFN-α co-treatment did not up-regulate Noxa, nor exerted any pro-apoptotic activity, in normal B-lymphocytes, suggesting that this effect is specific for lymphoma cells. In contrast to other compounds inducing Noxa-dependent MCL cell apoptosis, RA/IFN-α combination does not increase Mcl-1 protein levels, and even down-regulates A1/Bfl-1. Intriguingly, our results demonstrate that, under normal conditions, both Mcl-1 and A1/Bfl-1 can be bound to the full-length form of Bid, thus preventing its cleavage and repressing its activation. MCL cells exposure to RA/IFN-α combination relieves this repression through a competitive inhibition exerted by Noxa, which favors Bid displacement from pro-apoptotic/anti-apoptotic proteins complexes and its subsequent activation. Notably, we took advantage of multispectral imaging flow cytometry to selectively analyze Mcl-1-Bid and A1/Bfl-1-Bid co-localization in cells with morphometric features of early apoptosis, a distinction that is not usually feasible in co-immunoprecipitation experiments. This methodological approach is particularly relevant if we consider that the binding of Mcl-1 or A1/Bfl-1 to Bid abolishes its pro-apoptotic activity and that RA/IFN-α-induced Bid displacement from these complexes is an early event in the activation of apoptotic machinery.

Another relevant aspect of 9-*cis*-RA/IFN-α combination is the up-regulation of PLSCR1 in MCL cells. Originally described as a simple scramblase, PLSCR1 is now known to play a role also in cell signaling, maturation, apoptosis and growth of cancer cells [Zwaal *et al*., 2005; Huang *et al*., 2006]. Recent studies have revealed that PLSCR1 over-expression is associated with the differentiation of human myeloid leukemia cells into granulocytes [Nakamaki *et al*., 2002], and with the suppression of cell growth in ovarian carcinoma [Silverman *et al*, 2002]. Furthermore, Kuo *et al*. suggested that PLSCR1 could be both a novel diagnostic biomarker and an important prognostic factor for colorectal cancer [Kuo *et al*., 2011], and a correlation between PLSCR1 mRNA levels and improved overall survival was demonstrated in acute myeloid leukemia patients [Zhao *et al*., 2004]. As a potently stimulated IFN-responsive gene, PLSCR1 was also found to be required for the anti-viral/apoptotic activity of IFN, whereas PLSCR1 gene deletion and RNA-*interfering* suppression of PLSCR1 expression were found to inhibit the expression of a select subset of IFN-stimulated genes, including those with known anti-viral activity [Silverman *et al*., 2002; Dong *et al*., 2004]. Despite the evidence that PLSCR1 is an endofacial cell surface protein with apparent biologic function at the plasma membrane, recent data suggest an additional role for this protein in the nucleus, where it was found to bind directly to genomic DNA [Zhou *et al*., 2005]. Nuclear trafficking of PLSCR1 has been observed only in circumstances where its cellular expression was induced by IFN and other cytokines or growth factors that transcriptionally activate this gene, implying nuclear import of *de novo* synthesized PLSCR1 rather than a redistribution on the membrane compartments [Wiedmer *et al*., 2003]. These studies are particularly relevant if we consider that a potent induction of Noxa, at both mRNA and protein levels, was found in multiple human tumor cell lines after exposure to dsRNA and IFN, and that the protein is necessary to enhance their apoptotic/anti-viral activity. Importantly, Noxa regulation by IFN was independent of p53, the best known transcriptional factor of the Noxa gene PMAIP-1, thereby suggesting a novel mechanism of Noxa induction [Sun *et al*., 2005]. Taking into account the role of PLSCR1 in the apoptotic process and in increasing the transcriptional response to IFN, such evidence suggests that this protein may directly or indirectly induce the transcription of Noxa as an IFN-responsive gene, a topic that leaves room for further study. 9-*cis*-RA/IFN-α-induced PLSCR1 up-regulation occurs at both mRNA and protein levels in all MCL cell lines analyzed in our study. We investigated also the endogenous expression of PLSCR1 in 32 MCLs and this analysis demonstrated how this protein is heterogeneously expressed in this lymphoma. In particular, in 9 cases more than 10% of neoplastic cells express PLSCR1 and preliminary data showed how in these cases the proliferation index Ki-67 is significantly higher than in the negative ones. These findings are in

apparent contrast with our results showing that 9-*cis*-RA/IFN-α treatment induces a strong PLSCR1 expression coupled with a marked anti-proliferative activity in MCL cells. Furthermore, PLSCR1 silencing does not influence the accumulation of cells in G0-G1 promoted by 9-*cis*-RA/IFN-α exposure (not shown), indicating that, in MCL, this protein is mainly involved in apoptotic pathways. A possible explanation for this discrepancy may reside in the increased susceptibility to apoptosis of MCL cells with higher proliferation rates. In this respect, the heterogeneity of PLSCR1 expression, both in MCL tumor biopsies and cell lines, suggests that this protein could constitute a new biomarker for MCL classification, stimulating thus further studies to identify clinico-pathological correlations of possible diagnostic or prognostic relevance. As for Noxa, PLSCR1 silencing demonstrated how its expression is essential in 9-*cis*-RA/IFN-α-mediated apoptosis and contribute also to the apoptosis induced by different drugs currently employed in MCL management, like doxorubicin and bortezomib. In fact, although PLSCR1 expression is not up-regulated by these drugs when used alone, when its expression is previously induced by 9-*cis*-RA/IFN-α PLSCR1 is able to make MCL cells more responsive to the pro-apoptotic activity of both doxorubicin and bortezomib. Interestingly, suboptimal concentrations of doxorubicin or bortezomib unable to exert relevant pro-apoptotic effects if used alone, showed a significant synergistic activity in promoting cell death when added to the 9-*cis*-RA/IFN-α combination, and this effect is associated with a further increase of PLSCR1 as compared to the 9-*cis*-RA/IFN-α-only dependent up-regulation of the protein. These findings point out PLSCR1 as potential marker of susceptibility to different therapeutic drugs inducing apoptosis in MCL. We extended our analysis also to the monoclonal antibody rituximab commonly used in MCL management, demonstrating how 9-*cis*-RA/IFN-α pre-treatment does not sensitize MCL cells to rituximab anticancer activity. Notably, unlike doxorubicin and bortezomib, rituximab failed to enhance PLSCR1 expression when added in the medium of cells treated with 9-*cis*-RA/IFN-α. Moreover, the absence of any additive/synergistic effect between these drugs could be explained by the down-regulation of the CD20 expression induced by 9-*cis*-RA/IFN-α. Therefore, our data suggest that the use of rituximab in combination with 9-*cis*-RA/IFN-α is not advisable in the clinical practice. Preliminary data have shown that 9-*cis*-RA/IFN-α-promoted PLSCR1 induction is a clear effect even in FL and CTCL cell lines (not shown). The involvement of this protein in the apoptotic response to 9-*cis*-RA/IFN-α is under investigation also in these lymphoma histotypes. In future perspective, analysis of PLSCR1 expression might allow the identification of tumors more prone to undergo apoptosis, and strategies able to up-regulate PLSCR1, like RA/IFN-α combination, might successfully complement and improve conventional treatment modalities.

A deeper analysis of the mechanism underlying 9-*cis*-RA/IFN-α-induced PLSCR1 up-regulation allowed us to identify the STAT1 transcription factor as the main mediator of this event. Recently, it has been demonstrated that STAT1-dependent pathway is responsible, at least in part, of the MCL apoptotic response to interleukin-21, highlighting the possible therapeutic importance of this protein in this setting [Gelebart *et al*., 2009]. In the present study, we show that also 9-*cis*-RA/IFN-α combination relies on STAT1 activation to enhance MCL cell susceptibility to cell death by common anticancer drugs, thanks to a strong STAT1-dependent induction of PLSCR1 transcription. Moreover, our data show that 9-*cis*-RA/IFN-α-induced STAT1 activation, and the consequent PLSCR1 up-regulation, is the result of the triggering of Akt after a short-time exposure to the treatment. In fact, a time course analysis of Akt phosphorylation revealed how the activation of this kinase in MCL cells can be controlled by the treatment in a time-dependent manner, being activated at the beginning of the treatment and strongly inhibited after 24 hours of exposure. We found that 9-*cis*-RA/IFN-α-mediated apoptosis is associated with a strong inhibition of Akt and of its downstream kinase mTOR in MCL, FL, and CTCL cell lines. Up-regulation of the pro-apoptotic protein Noxa occurred in all the different lymphoma histotypes studied and, in particular, it was observed in MCL cell lines carrying either wild-type or mutant p53, further supporting the hypothesis that transcription factors different from p53 may be involved in this phenomenon. Recent studies revealed a conserved FOXO-binding site in the NOXA promoter and, using siRNA knock-down, a specific role for the FOXO family proteins in activating NOXA transcription in cancer cells was identified [Valis *et al*., 2011]. Analysis of the phosphorylated forms of FOXO3a transcription factor, a downstream target of Akt, in lymphoma cell lines treated with 9-*cis*-RA/IFN-α showed a de-phosphorylation/activation of the protein at Ser318/321 that results in FOXO3a nuclear translocation, as a consequence of Akt inhibition. The results presented herein support the conclusion that the down-regulation of the Akt pathway by RA/IFN-α, resulting in FOXO3a de-phosphorylation/activation and its subsequent nuclear internalization followed by Noxa up-regulation, is one of the main molecular mechanisms underlying RA/IFN-α-dependent Akt-driven lymphoma cell death. Notably, specific pharmacologic inhibition of Akt and TORC1 in FL cell lines indicated that suppression of Akt, but not of mTOR, induces apoptosis, consistently with the findings by Bhende *et al*., Marzec *et al*. for CTCL, and with our previous findings in MCL [Marzec *et al*., 2008; Dal Col *et al*. 2008; Bhende *et al*., 2010]. These findings point out that an Akt substrate different from mTOR is likely involved in mediating its pro-survival effect in lymphoma cells, and suggest that the mechanism of 9-*cis*-RA/IFN-α-induced cell death in lymphoid malignancies mainly depends on Akt but not on mTOR inhibition. A large part of the

data discussed above are publisched in the following manuscript: *Dal Col J, **Mastorci K**, Faè DA, Muraro E, Martorelli D, Inghirami G, et al.* Retinoic acid/alpha-interferon combination inhibits growth and promotes apoptosis in mantle cell lymphoma through Akt-dependent modulation of critical targets. Cancer Res. 2012 Apr 1;72(7):1825-1835.

Many recent findings have highlighted the important function of autophagy in cancer cells and how this process is molecularly interwoven with the apoptotic machinery [Maiuri *et al*., 2007; Vicencio *et al*., 2008; Moscat *et al*., 2009]. Autophagy is a type of cellular catabolic degradation response to nutrient starvation or metabolic stress. Its main function is to maintain intracellular metabolic homeostasis through degradation of unfolded or aggregated proteins and organelles, in parallel with the ubiquitin proteasome degradation pathway [Mathew *et al*., 2007]. In addition to this key function, this biological process was also found to be responsible for other important functions, especially under stressful situation [Chen *et al*., 2010]. In fact, autophagy was initially believed as a non-apoptotic programme of cell death, or "type-II" cell death, to distinguish it from apoptosis [Rami *et al*., 2009]. However, under most circumstances, autophagy promotes cell survival by adapting cells to the stress conditions, which is functionally paradoxical to apoptosis [Rami *et al*., 2009]. This evidence made it fundamentally important to clarify whether autophagy is a main strategy for cell survival, or if it also serves as a trigger for cell death. Although it is still controversial whether autophagy kills cancer cells or sustains their survival under stressful conditions, more and more reports provide data to support that autophagy promotes cancer cell survival after chemotherapy or radiation therapy [Carew *et al*., 2007; Apel *et al*., 2008; Chen *et al*., 2009]. For example, autophagy facilitates the resistance of chronic myeloid leukemia (CML) to Imatinib [Bellodi *et al*., 2009], and also potentiates the resistance of HER2 positive breast cancer cells to anti-HER2 monoclonal antibody trastuzumab [Vazquez-Martin *et al*., 2009]. Intriguingly, abrogation of autophagy by selective inhibitors, such as 3-methyladenine, chloroquine or bafilomycin, or by shRNA knockdown of autophagy-related molecules, re-sensitizes resistant cancer cells to chemotherapy or radiation [Apel *et al*., 2008; Bellodi *et al*., 2009]. It is also noteworthy that the autophagic inhibitor hydroxyl-chloroquine has already been applied in a clinical trial [Chen *et al*., 2010]. These findings highlight the possible therapeutic potential of autophagy inhibition in cancer cells, thus making it likely to expect autophagic inhibitors to be the next generation of drugs to overcome anti-cancer therapies resistance. mTOR protein is a well known antagonist of the autophagic process and, as its upstream regulator, Akt is then able to control/inhibit autophagy through mTOR activating phosphorylation [Sobolewska *et al*., 2009; Qin *et al*., 2010; Choi *et al*., 2010]. Considering the

ability of 9-*cis*-RA/IFN-α combination to efficiently inactivate Akt, we recently focused our efforts on the study of the possible involvement of autophagy in 9-*cis*-RA/IFN-α-induced cell death. Preliminary data have brought to light that the exposure of MCL, FL, and CTCL cell lines to 9-*cis*-RA/IFN-α increases the formation of lysosomal and autophagic vesicles and enhances the LC3II/LC3I ratio together with Beclin-1 up-regulation, two known autophagic markers (not shown). Moreover, the levels of p62/SQSTM1, a ubiquitin binding protein involved in the cellular delivery of protein aggregates to the autophagosome [Pankiv *et al*., 2007] decrease after the treatment, as a consequence of the enhanced lysosomal-dependent degradation of autophagosomes during autophagy (not shown). Noteworthy, treatment induced-Beclin-1 up-regulation was observed also in primary MCL cells (not shown). Intriguingly, using TORC1 and Akt specific inhibitors, we demonstrated that 9-*cis*-RA/IFN-α-induced autophagy is efficiently mediated by Akt inhibition, but, unlike the 9-*cis*-RA/IFN-α-dependent apoptotic process, it occurs through mTOR inactivation (not shown). We are currently investigating whether 9-*cis*-RA/IFN-α-induced autophagy is a compensatory survival mechanism triggered by the cell to withstand the apoptotic effect of the treatment or a synergistic mechanism of cell death.

Overall, these findings further highlight the critical relevance of the PI3-K/Akt pathway in lymphomas and suggest that targeting Akt may result in more pronounced and effective therapeutic effects as compared with a selective mTOR inhibition. Considering that 9-*cis*-RA/IFN-α combination efficiently inactivates Akt and is associated with a lower toxicity compared to other Akt inhibitors, our results provide a strong rationale to re-assess the potential clinical relevance of these well-known and relatively cheap drugs in the management of poorly responsive Akt-driven non-Hodgkin lymphomas.

BIBLIOGRAFY

1. Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, et al. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. Proc. Natl. Acad. Sci. U.S.A. 1993 Gen 1;90(1):30-34.

2. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat. Rev. Cancer. 2001 Dic;1(3):181-193.

3. Ambinder RF. Mystery of the missing target. Blood. 2010 Nov 11;116(19):3691-3692.

4. Anderson JR, Armitage JO, Weisenburger DD. Epidemiology of the non-Hodgkin's lymphomas: distributions of the major subtypes differ by geographic locations. Non-Hodgkin's Lymphoma Classification Project. Ann. Oncol. 1998 Lug;9(7):717-720.

5. Ansell SM, Inwards DJ, Rowland KM, Flynn PJ, Morton RF, Moore DF, et al. Low-dose, single-agent temsirolimus for relapsed mantle cell lymphoma: a phase 2 trial in the North Central Cancer Treatment Group. Cancer. 2008 Ago 1;113(3):508-514.

6. Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res. 2008 Mar 1;68(5):1485-1494.

7. Balasubramanian K, Mirnikjoo B, Schroit AJ. Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. J. Biol. Chem. 2007 Giu 22;282(25):18357-18364.

8. Balkwill FR, Burke F. The cytokine network. Immunol. Today. 1989 Set;10(9):299-304.

9. Baron S, Tyring SK, Fleischmann WR, Coppenhaver DH, Niesel DW, Klimpel GR, et al. The interferons. Mechanisms of action and clinical applications. JAMA. 1991 Set 11;266(10):1375-1383.

10. Becknell B, Trotta R, Yu J, Ding W, Mao HC, Hughes T, et al. Efficient infection of human natural killer cells with an EBV/retroviral hybrid vector. J. Immunol. Methods. 2005 Gen;296(1-2):115-123.

11. Bellodi C, Lidonnici MR, Hamilton A, Helgason GV, Soliera AR, Ronchetti M, et al. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. J. Clin. Invest. 2009 Mag;119(5):1109-1123.

12. Beum PV, Lindorfer MA, Hall BE, George TC, Frost K, Morrissey PJ, et al. Quantitative analysis of protein co-localization on B cells opsonized with rituximab and complement using the ImageStream multispectral imaging flow cytometer. J. Immunol. Methods. 2006 Dic 20;317(1-2):90-99.

13. Bhatti Z, Berenson CS. Adult systemic cat scratch disease associated with therapy for hepatitis C. BMC Infect. Dis. 2007;7:8.

14. Bhende PM, Park SI, Lim MS, Dittmer DP, Damania B. The dual PI3K/mTOR inhibitor,

NVP-BEZ235, is efficacious against follicular lymphoma. Leukemia. 2010 Ott;24(10):1781-1784.

15. Bjornsti M, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat. Rev. Cancer. 2004 Mag;4(5):335-348.

16. Bollag W, Holdener EE. Retinoids in cancer prevention and therapy. Ann. Oncol. 1992 Lug;3(7):513-526.

17. Bunney TD, Katan M. Phosphoinositide signalling in cancer: beyond PI3K and PTEN. Nat. Rev. Cancer. 2010 Mag;10(5):342-352.

18. Campo E, Raffeld M, Jaffe ES. Mantle-cell lymphoma. Semin. Hematol. 1999 Apr;36(2):115-127.

19. Caraglia M, Marra M, Pelaia G, Maselli R, Caputi M, Marsico SA, et al. Alpha-interferon and its effects on signal transduction pathways. J. Cell. Physiol. 2005 Feb;202(2):323-335.

20. Carew JS, Nawrocki ST, Cleveland JL. Modulating autophagy for therapeutic benefit. Autophagy. 2007 Ott;3(5):464-467.

21. Chawla-Sarkar M, Lindner DJ, Liu Y, Williams BR, Sen GC, Silverman RH, et al. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. Apoptosis. 2003 Giu;8(3):237-249.

22. Chen N, Debnath J. Autophagy and tumorigenesis. FEBS Lett. 2010 Apr 2;584(7):1427-1435.

23. Chen M, Ben-Efraim I, Mitrousis G, Walker-Kopp N, Sims PJ, Cingolani G. Phospholipid scramblase 1 contains a nonclassical nuclear localization signal with unique binding site in importin alpha. J. Biol. Chem. 2005 Mar 18;280(11):10599-10606.

24. Chen S, Rehman SK, Zhang W, Wen A, Yao L, Zhang J. Autophagy is a therapeutic target in anticancer drug resistance. Biochim. Biophys. Acta. 2010 Dic;1806(2):220-229.

25. Cheung Y, Lau WK, Yu M, Lai CS, Yeung S, So K, et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology. 2009 Gen;30(1):127-135.

26. Chiarini F, Del Sole M, Mongiorgi S, Gaboardi GC, Cappellini A, Mantovani I, et al. The novel Akt inhibitor, perifosine, induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrug-resistant human T-acute leukemia cells by a JNK-dependent mechanism. Leukemia. 2008 Giu;22(6):1106-1116.

27. Chiarle R, Budel LM, Skolnik J, Frizzera G, Chilosi M, Corato A, et al. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. Blood. 2000 Gen 15;95(2):619-626.

28. Choi K, Kim S, Ha J, Kim S, Son JH. A novel mTOR activating protein protects dopamine neurons against oxidative stress by repressing autophagy related cell death. J. Neurochem. 2010 Gen;112(2):366-376.

29. Coiffier B, Ribrag V. Exploring mammalian target of rapamycin (mTOR) inhibition for treatment of mantle cell lymphoma and other hematologic malignancies. Leuk. Lymphoma. 2009 Dic;50(12):1916-1930.

30. Dal Col J, Dolcetti R. GSK-3beta inhibition: at the crossroad between Akt and mTOR constitutive activation to enhance cyclin D1 protein stability in mantle cell lymphoma. Cell Cycle. 2008 Set 15;7(18):2813-2816.

31. Dal Col J, Zancai P, Terrin L, Guidoboni M, Ponzoni M, Pavan A, et al. Distinct functional significance of Akt and mTOR constitutive activation in mantle cell lymphoma. Blood. 2008 Mag 15;111(10):5142-5151.

32. Dalla-Favera R. Lymphoid malignancies: many tumor types, many altered genes, many therapeutic challenges. J. Clin. Invest. 2012 Ott 1;122(10):3396-3397.

33. Darnell JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994 Giu 3;264(5164):1415-1421.

34. Decker T, Meinke A. Jaks, Stats and the immune system. Immunobiology. 1997 Dic;198(1-3):99-111.

35. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 1998 Nov 15;12(22):3499-3511.

36. Dong B, Zhou Q, Zhao J, Zhou A, Harty RN, Bose S, et al. Phospholipid scramblase 1 potentiates the antiviral activity of interferon. J. Virol. 2004 Set;78(17):8983-8993.

37. Drakos E, Rassidakis GZ, Medeiros LJ. Mammalian target of rapamycin (mTOR) pathway signalling in lymphomas. Expert Rev Mol Med. 2008;10:e4.

38. Dunleavy K, Piekarz RL, Zain J, Janik JE, Wilson WH, O'Connor OA, et al. New strategies in peripheral T-cell lymphoma: understanding tumor biology and developing novel therapies. Clin. Cancer Res. 2010 Dic 1;16(23):5608-5617.

39. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell. 1996 Feb 9;84(3):443-450.

40. Fasolo A, Sessa C. Current and future directions in mammalian target of rapamycin inhibitors development. Expert Opin Investig Drugs. 2011 Mar;20(3):381-394.

41. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, Ruggero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol. 2009 Feb 10;7(2):e38.

42. Fitzpatrick JE, Mellette JR. Treatment of mycosis fungoides with isotretinoin. J Dermatol Surg Oncol. 1986 Giu;12(6):626-629.

43. Flinterman M, Guelen L, Ezzati-Nik S, Killick R, Melino G, Tominaga K, et al. E1A activates transcription of p73 and Noxa to induce apoptosis. J. Biol. Chem. 2005 Feb 18;280(7):5945-5959.

44. Gelebart P, Zak Z, Anand M, Dien-Bard J, Amin HM, Lai R. Interleukin-21 effectively induces apoptosis in mantle cell lymphoma through a STAT1-dependent mechanism. Leukemia. 2009 Ott;23(10):1836-1846.

45. George TC, Fanning SL, Fitzgerald-Bocarsly P, Fitzgeral-Bocarsly P, Medeiros RB, Highfill S, et al. Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. J. Immunol. Methods. 2006 Apr 20;311(1-2):117-129.

46. Girardi M, Heald PW, Wilson LD. The pathogenesis of mycosis fungoides. N. Engl. J. Med. 2004 Mag 6;350(19):1978-1988.

47. Glass AG, Karnell LH, Menck HR. The National Cancer Data Base report on non-Hodgkin's lymphoma. Cancer. 1997 Dic 15;80(12):2311-2320.

48. Greiner TC, Dasgupta C, Ho VV, Weisenburger DD, Smith LM, Lynch JC, et al. Mutation and genomic deletion status of ataxia telangiectasia mutated (ATM) and p53 confer specific gene expression profiles in mantle cell lymphoma. Proc. Natl. Acad. Sci. U.S.A. 2006 Feb 14;103(7):2352-2357.

49. Guidoboni M, Zancai P, Cariati R, Rizzo S, Dal Col J, Pavan A, et al. Retinoic acid inhibits the proliferative response induced by CD40 activation and interleukin-4 in mantle cell lymphoma. Cancer Res. 2005 Gen 15;65(2):587-595.

50. Gulmann C, Espina V, Petricoin E, Longo DL, Santi M, Knutsen T, et al. Proteomic analysis of apoptotic pathways reveals prognostic factors in follicular lymphoma. Clin. Cancer Res. 2005 Ago 15;11(16):5847-5855.

51. Gupta M, Dillon SR, Ziesmer SC, Feldman AL, Witzig TE, Ansell SM, et al. A proliferation-inducing ligand mediates follicular lymphoma B-cell proliferation and cyclin D1 expression through phosphatidylinositol 3-kinase-regulated mammalian target of rapamycin activation. Blood. 2009 Mag 21;113(21):5206-5216.

52. Hart JR, Liao L, Yates JR, Vogt PK. Essential role of Stat3 in PI3K-induced oncogenic transformation. Proc. Natl. Acad. Sci. U.S.A. 2011 Ago 9;108(32):13247-13252.

53. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004 Ago 15;18(16):1926-1945.

54. Hershko T, Ginsberg D. Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. J. Biol. Chem. 2004 Mar 5;279(10):8627-8634.

55. Hess G, Herbrecht R, Romaguera J, Verhoef G, Crump M, Gisselbrecht C, et al. Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. J. Clin. Oncol. 2009 Ago 10;27(23):3822-3829.

56. Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhoa L, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood. 1988 Ago;72(2):567-572.

57. Huang Y, Zhao Q, Chen G. Phospholipid scramblase 1. Sheng Li Xue Bao. 2006 Dic 25;58(6):501-510.

58. Hwang ST, Janik JE, Jaffe ES, Wilson WH. Mycosis fungoides and Sézary syndrome. Lancet. 2008 Mar 15;371(9616):945-957.

59. Jadayel DM, Lukas J, Nacheva E, Bartkova J, Stranks G, De Schouwer PJ, et al. Potential role for concurrent abnormalities of the cyclin D1, p16CDKN2 and p15CDKN2B genes in certain B cell non-Hodgkin's lymphomas. Functional studies in a cell line (Granta 519). Leukemia. 1997 Gen;11(1):64-72.

60. Jones RJ, Chen Q, Voorhees PM, Young KH, Bruey-Sedano N, Yang D, et al. Inhibition of the p53 E3 ligase HDM-2 induces apoptosis and DNA damage--independent p53 phosphorylation in mantle cell lymphoma. Clin. Cancer Res. 2008 Set 1;14(17):5416-5425.

61. Katsoulidis E, Li Y, Yoon P, Sassano A, Altman J, Kannan-Thulasiraman P, et al. Role of the p38 mitogen-activated protein kinase pathway in cytokine-mediated hematopoietic suppression in myelodysplastic syndromes. Cancer Res. 2005 Ott 1;65(19):9029-9037.

62. Kaur S, Lal L, Sassano A, Majchrzak-Kita B, Srikanth M, Baker DP, et al. Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. J. Biol. Chem. 2007 Gen 19;282(3):1757-1768.

63. Klier M, Anastasov N, Hermann A, Meindl T, Angermeier D, Raffeld M, et al. Specific lentiviral shRNA-mediated knockdown of cyclin D1 in mantle cell lymphoma has minimal effects on cell survival and reveals a regulatory circuit with cyclin D2. Leukemia. 2008 Nov;22(11):2097-2105.

64. Kovarik P, Mangold M, Ramsauer K, Heidari H, Steinborn R, Zotter A, et al. Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression. EMBO J. 2001 Gen 15;20(1-2):91-100.

65. Kuo Y, Chan C, Chang CA, Fan C, Hung R, Hung Y, et al. Identification of phospholipid scramblase 1 as a biomarker and determination of its prognostic value for colorectal cancer. Mol. Med. 2011 Feb;17(1-2):41-47.

66. Lannutti BJ, Meadows SA, Herman SEM, Kashishian A, Steiner B, Johnson AJ, et al. CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood. 2011 Gen 13;117(2):591-594.

67. Lansigan F, Foss FM. Current and emerging treatment strategies for cutaneous T-cell lymphoma. Drugs. 2010 Feb 12;70(3):273-286.

68. Lansigan F, Choi J, Foss FM. Cutaneous T-cell lymphoma. Hematol. Oncol. Clin. North Am. 2008 Ott;22(5):979-996, x.

69. Lei H, Furlong PJ, Ra JH, Mullins D, Cantor R, Fraker DL, et al. AKT activation and response to interferon-beta in human cancer cells. Cancer Biol. Ther. 2005 Lug;4(7):709-715.

70. Lenz G, Dreyling M. Does the combination of rituximab and thalidomide influence the long-term perspectives of advanced-stage MCL? Nat Clin Pract Oncol. 2005 Feb;2(2):72-73.

71. Lenz G, Staudt LM. Aggressive lymphomas. N. Engl. J. Med. 2010 Apr 15;362(15):1417-1429.

72. Leseux L, Hamdi SM, Al Saati T, Capilla F, Recher C, Laurent G, et al. Syk-dependent mTOR activation in follicular lymphoma cells. Blood. 2006 Dic 15;108(13):4156-4162.

73. Liu M, Iavarone A, Freedman LP. Transcriptional activation of the human p21(WAF1/CIP1) gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. J. Biol. Chem. 1996 Dic 6;271(49):31723-31728.

74. Lotan R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochim. Biophys. Acta. 1980 Mar 12;605(1):33-91.

75. Luo Y, Shoemaker AR, Liu X, Woods KW, Thomas SA, de Jong R, et al. Potent and selective inhibitors of Akt kinases slow the progress of tumors in vivo. Mol. Cancer Ther. 2005 Giu;4(6):977-986.

76. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat. Rev. Mol. Cell Biol. 2007 Set;8(9):741-752.

77. Martínez N, Camacho FI, Algara P, Rodríguez A, Dopazo A, Ruíz-Ballesteros E, et al. The molecular signature of mantle cell lymphoma reveals multiple signals favoring cell survival. Cancer Res. 2003 Dic 1;63(23):8226-8232.

78. Marzec M, Halasa K, Kasprzycka M, Wysocka M, Liu X, Tobias JW, et al. Differential effects of interleukin-2 and interleukin-15 versus interleukin-21 on CD4+ cutaneous T-cell lymphoma cells. Cancer Res. 2008 Feb 15;68(4):1083-1091.

79. Marzec M, Liu X, Wysocka M, Rook AH, Odum N, Wasik MA. Simultaneous inhibition of mTOR-containing complex 1 (mTORC1) and MNK induces apoptosis of cutaneous T-cell lymphoma (CTCL) cells. PLoS ONE. 2011;6(9):e24849.

80. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat. Rev. Cancer. 2007 Dic;7(12):961-967.

81. Mayer H, Bollag W, Hänni R, Rüegg R. Retinoids, a new class of compounds with prophylactic and therapeutic activities in oncology and dermatology. Experientia. 1978 Set 15;34(9):1105-1119.

82. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature. 2000 Apr 13;404(6779):782-787.

83. Miller VA, Rigas JR, Benedetti FM, Verret AL, Tong WP, Kris MG, et al. Initial clinical trial of the retinoid receptor pan agonist 9-cis retinoic acid. Clin. Cancer Res. 1996 Mar;2(3):471-475.

84. Mills GB, Lu Y, Fang X, Wang H, Eder A, Mao M, et al. The role of genetic abnormalities of PTEN and the phosphatidylinositol 3-kinase pathway in breast and ovarian tumorigenesis, prognosis, and therapy. Semin. Oncol. 2001 Ott;28(5 Suppl 16):125-141.

85. Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. Cell. 2009 Giu 12;137(6):1001-1004.

86. Moynihan MJ, Bast MA, Chan WC, Delabie Jan, Wickert RS, Wu G, et al. Lymphomatous polyposis. A neoplasm of either follicular mantle or germinal center cell origin. Am. J. Surg. Pathol. 1996 Apr;20(4):442-452.

87. Nakamaki T, Okabe-Kado J, Yamamoto-Yamaguchi Y, Hino KI, Tomoyasu S, Honma Y, et al. Role of MmTRA1b/phospholipid scramblase1 gene expression in the induction of differentiation of human myeloid leukemia cells into granulocytes. Exp. Hematol. 2002 Mag;30(5):421-429.

88. Navarro A, Anand-Apte B, Tanabe Y, Feldman G, Larner AC. A PI-3 kinase-dependent, Stat1-independent signaling pathway regulates interferon-stimulated monocyte adhesion. J. Leukoc. Biol. 2003 Apr;73(4):540-545.

89. Nguyen H, Ramana CV, Bayes J, Stark GR. Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. J. Biol. Chem. 2001 Set 7;276(36):33361-33368.

90. Nickoloff BJ, Basham TY, Merigan TC, Morhenn VB. Immunomodulatory and antiproliferative effect of recombinant alpha, beta, and gamma interferons on cultured human malignant squamous cell lines, SCL-1 and SW-1271. J. Invest. Dermatol. 1985 Giu;84(6):487-490.

91. Obexer P, Geiger K, Ambros PF, Meister B, Ausserlechner MJ. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. Cell Death Differ. 2007 Mar;14(3):534-547.

92. O'Reilly KE, Rojo F, She Q, Solit D, Mills GB, Smith D, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. 2006 Feb 1;66(3):1500-1508.

93. Pal SK, Reckamp K, Yu H, Figlin RA. Akt inhibitors in clinical development for the treatment of cancer. Expert Opin Investig Drugs. 2010 Nov;19(11):1355-1366.

94. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun J, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. 2007 Ago 17;282(33):24131-24145.

95. Parmar S, Platanias LC. Interferons: mechanisms of action and clinical applications. Curr Opin Oncol. 2003 Nov;15(6):431-439.

96. Peponi E, Drakos E, Reyes G, Leventaki V, Rassidakis GZ, Medeiros LJ. Activation of mammalian target of rapamycin signaling promotes cell cycle progression and protects cells from apoptosis in mantle cell lymphoma. Am. J. Pathol. 2006 Dic;169(6):2171-2180.

97. Pérez-Galán P, Roué G, Villamor N, Campo E, Colomer D. The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. Blood. 2007 Mag 15;109(10):4441-4449.

98. Pérez-Galán P, Dreyling M, Wiestner A. Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. Blood. 2011 Gen 6;117(1):26-38.

99. Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. Annu. Rev. Biochem. 1987;56:727-777.

100. Pettersson F, Dalgleish AG, Bissonnette RP, Colston KW. Retinoids cause apoptosis in pancreatic cancer cells via activation of RAR-gamma and altered expression of Bcl-2/Bax. Br. J. Cancer. 2002 Ago 27;87(5):555-561.

101. Pfeffer LM, Dinarello CA, Herberman RB, Williams BR, Borden EC, Bordens R, et al. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. Cancer Res. 1998 Giu 15;58(12):2489-2499.

102. Piya S, Moon AR, Song PI, Hiscott J, Lin R, Seol D, et al. Suppression of IRF4 by IRF1, 3, and 7 in Noxa expression is a necessary event for IFN-γ-mediated tumor elimination. Mol. Cancer Res. 2011 Ott;9(10):1356-1365.

103. Platanias LC. Map kinase signaling pathways and hematologic malignancies. Blood. 2003 Giu 15;101(12):4667-4679.

104. Pomponi F, Cariati R, Zancai P, De Paoli P, Rizzo S, Tedeschi RM, et al. Retinoids irreversibly inhibit in vitro growth of Epstein-Barr virus-immortalized B lymphocytes. Blood. 1996 Ott 15;88(8):3147-3159.

105. Qin A, Liu C, Qin Y, Hong L, Xu M, Yang L, et al. Autophagy was activated in injured astrocytes and mildly decreased cell survival following glucose and oxygen deprivation and focal cerebral ischemia. Autophagy. 2010 Ago;6(6):738-753.

106. Rami A. Review: autophagy in neurodegeneration: firefighter and/or incendiarist? Neuropathol. Appl. Neurobiol. 2009 Ott;35(5):449-461.

107. Rieger AM, Hall BE, Luong LT, Schang LM, Barreda DR. Conventional apoptosis assays using propidium iodide generate a significant number of false positives that prevent accurate assessment of cell death. J. Immunol. Methods. 2010 Giu 30;358(1-2):81-92.

108. Rudelius M, Pittaluga S, Nishizuka S, Pham TH, Fend F, Jaffe ES, et al. Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. Blood. 2006 Set 1;108(5):1668-1676.

109. Sahu SK, Gummadi SN, Manoj N, Aradhyam GK. Phospholipid scramblases: an overview. Arch. Biochem. Biophys. 2007 Giu 1;462(1):103-114.

110. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science. 2004 Apr 23;304(5670):554.

111. Schatz JH. Targeting the PI3K/AKT/mTOR pathway in non-Hodgkin's lymphoma: results, biology, and development strategies. Curr Oncol Rep. 2011 Ott;13(5):398-406.

112. Shankland KR, Armitage JO, Hancock BW. Non-Hodgkin lymphoma. Lancet. 2012 Set 1;380(9844):848-857.

113. Sidell N. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. J. Natl. Cancer Inst. 1982 Apr;68(4):589-596.

114. Silverman RH, Halloum A, Zhou A, Dong B, Al-Zoghaibi F, Kushner D, et al. Suppression of ovarian carcinoma cell growth in vivo by the interferon-inducible plasma membrane protein, phospholipid scramblase 1. Cancer Res. 2002 Gen 15;62(2):397-402.

115. Smith SM, van Besien K, Karrison T, Dancey J, McLaughlin P, Younes A, et al. Temsirolimus has activity in non-mantle cell non-Hodgkin's lymphoma subtypes: The University of Chicago phase II consortium. J. Clin. Oncol. 2010 Nov 1;28(31):4740-4746.

116. Sobolewska A, Gajewska M, Zarzyńska J, Gajkowska B, Motyl T. IGF-I, EGF, and sex steroids regulate autophagy in bovine mammary epithelial cells via the mTOR pathway. Eur. J. Cell Biol. 2009 Feb;88(2):117-130.

117. Spinella MJ, Freemantle SJ, Sekula D, Chang JH, Christie AJ, Dmitrovsky E. Retinoic acid promotes ubiquitination and proteolysis of cyclin D1 during induced tumor cell differentiation. J. Biol. Chem. 1999 Lug 30;274(31):22013-22018.

118. Stefancikova L, Moulis M, Fabian P, Ravcukova B, Vasova I, Muzik J, et al. Loss of the p53 tumor suppressor activity is associated with negative prognosis of mantle cell lymphoma. Int. J. Oncol. 2010 Mar;36(3):699-706.

119. Sun Y, Leaman DW. Ectopic expression of toll-like receptor-3 (TLR-3) overcomes the double-stranded RNA (dsRNA) signaling defects of P2.1 cells. J. Interferon Cytokine Res. 2004 Giu;24(6):350-361.

120. Sun Y, Leaman DW. Involvement of Noxa in cellular apoptotic responses to interferon, double-stranded RNA, and virus infection. J. Biol. Chem. 2005 Apr 22;280(16):15561-15568.

121. Sundaresan A, Claypool K, Mehta K, Lopez-Berestein G, Cabanillas F, Ford RJ. Retinoid-mediated inhibition of cell growth with stimulation of apoptosis in aggressive B-cell lymphomas. Cell Growth Differ. 1997 Ott;8(10):1071-1082.

122. Swenson WT, Wooldridge JE, Lynch CF, Forman-Hoffman VL, Chrischilles E, Link BK. Improved survival of follicular lymphoma patients in the United States. J. Clin. Oncol. 2005 Ago 1;23(22):5019-5026.

123. Tabe Y, Sebasigari D, Jin L, Rudelius M, Davies-Hill T, Miyake K, et al. MDM2 antagonist nutlin-3 displays antiproliferative and proapoptotic activity in mantle cell lymphoma. Clin. Cancer Res. 2009 Feb 1;15(3):933-942.

124. Tay K, Dunleavy K, Wilson WH. Novel agents for B-cell non-Hodgkin lymphoma: science and the promise. Blood Rev. 2010 Mar;24(2):69-82.

125. Tzivion G, Dobson M, Ramakrishnan G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. Biochim. Biophys. Acta. 2011 Nov;1813(11):1938-1945.

126. Uddin S, Sassano A, Deb DK, Verma A, Majchrzak B, Rahman A, et al. Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. J. Biol. Chem. 2002 Apr 26;277(17):14408-14416.

127. Valis K, Prochazka L, Boura E, Chladova J, Obsil T, Rohlena J, et al. Hippo/Mst1 stimulates transcription of the proapoptotic mediator NOXA in a FoxO1-dependent manner. Cancer Res. 2011 Feb 1;71(3):946-954.

128. Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail regulates protein stability and function. Mol. Cell. Biol. 2000 Lug;20(14):5010-5018.

129. Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. PLoS ONE. 2009;4(7):e6251.

130. Vicencio JM, Galluzzi L, Tajeddine N, Ortiz C, Criollo A, Tasdemir E, et al. Senescence, apoptosis or autophagy? When a damaged cell must decide its path--a mini-review. Gerontology. 2008;54(2):92-99.

131. Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, et al. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat. Med. 2002 Ott;8(10):1136-1144.

132. Visser HP, Tewis M, Willemze R, Kluin-Nelemans JC. Mantle cell lymphoma proliferates upon IL-10 in the CD40 system. Leukemia. 2000 Ago;14(8):1483-1489.

133. Visser HP, Tewis M, Willemze R, Kluin-Nelemans JC. Mantle cell lymphoma proliferates upon IL-10 in the CD40 system. Leukemia. 2000 Ago;14(8):1483-1489.

134. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat. Rev. Cancer. 2002 Lug;2(7):489-501.

135. Wang X, Sun S. Enhancing mTOR-targeted cancer therapy. Expert Opin. Ther. Targets. 2009 Ott;13(10):1193-1203.

136. Weinstock-Guttman B, Jacobs LD. What is new in the treatment of multiple sclerosis? Drugs. 2000 Mar;59(3):401-410.

137. Wen Z, Zhong Z, Darnell JE. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell. 1995 Lug 28;82(2):241-250.

138. Wendel H, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature. 2004 Mar 18;428(6980):332-337.

139. Wiedmer T, Zhao J, Nanjundan M, Sims PJ. Palmitoylation of phospholipid scramblase 1 controls its distribution between nucleus and plasma membrane. Biochemistry. 2003 Feb 11;42(5):1227-1233.

140. Witzig TE, Geyer SM, Ghobrial I, Inwards DJ, Fonseca R, Kurtin P, et al. Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. J. Clin. Oncol. 2005 Ago 10;23(23):5347-5356.

141. Witzig TE, Reeder CB, LaPlant BR, Gupta M, Johnston PB, Micallef IN, et al. A phase II trial of the oral mTOR inhibitor everolimus in relapsed aggressive lymphoma. Leukemia. 2011 Feb;25(2):341-347.

142. Yang CH, Murti A, Pfeffer SR, Kim JG, Donner DB, Pfeffer LM. Interferon alpha /beta promotes cell survival by activating nuclear factor kappa B through phosphatidylinositol 3-kinase and Akt. J. Biol. Chem. 2001 Apr 27;276(17):13756-13761.

143. Zancai P, Cariati R, Rizzo S, Boiocchi M, Dolcetti R. Retinoic acid-mediated growth arrest of EBV-immortalized B lymphocytes is associated with multiple changes in G1 regulatory proteins: p27Kip1 up-regulation is a relevant early event. Oncogene. 1998 Ott 8;17(14):1827-1836.

144. Zancai P, Dal Col J, Piccinin S, Guidoboni M, Cariati R, Rizzo S, et al. Retinoic acid stabilizes p27Kip1 in EBV-immortalized lymphoblastoid B cell lines through enhanced proteasome-dependent degradation of the p45Skp2 and Cks1 proteins. Oncogene. 2005 Apr 7;24(15):2483-2494.

145. Zha H, Raffeld M, Charboneau L, Pittaluga S, Kwak LW, Petricoin E, et al. Similarities of prosurvival signals in Bcl-2-positive and Bcl-2-negative follicular lymphomas identified by reverse phase protein microarray. Lab. Invest. 2004 Feb;84(2):235-244.

146. Zhang L, Li J, Xu W. A review of the role of Puma, Noxa and Bim in the tumorigenesis, therapy and drug resistance of chronic lymphocytic leukemia. Cancer Gene Ther. 2013 Gen;20(1):1-7.

147. Zhao K, Li X, Zhao Q, Huang Y, Li D, Peng Z, et al. Protein kinase Cdelta mediates retinoic acid and phorbol myristate acetate-induced phospholipid scramblase 1 gene expression: its role in leukemic cell differentiation. Blood. 2004 Dic 1;104(12):3731-3738.

148. Zhao K, Li D, Zhao Q, Huang Y, Silverman RH, Sims PJ, et al. Interferon-alpha-induced expression of phospholipid scramblase 1 through STAT1 requires the sequential activation of protein kinase Cdelta and JNK. J. Biol. Chem. 2005 Dic 30;280(52):42707-42714.

149. Zhou Q, Zhao J, Al-Zoghaibi F, Zhou A, Wiedmer T, Silverman RH, et al. Transcriptional control of the human plasma membrane phospholipid scramblase 1 gene is mediated by interferon-alpha. Blood. 2000 Apr 15;95(8):2593-2599.

150. Zhou Q, Ben-Efraim I, Bigcas J, Junqueira D, Wiedmer T, Sims PJ. Phospholipid scramblase 1 binds to the promoter region of the inositol 1,4,5-triphosphate receptor type 1 gene to enhance its expression. J. Biol. Chem. 2005 Ott 14;280(41):35062-35068.

151. Zwaal RFA, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. Cell. Mol. Life Sci. 2005 Mag;62(9):971-988.

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fosse davvero un "buon non-compleanno"!