**ROMANIAN ACADEMY**

**Institute of Biochemistry**

# *SUMMARY* OF THE PHD THESIS:

# NOVEL REGULATORY/MODULATORY MECHANISMS OF FOXO3/FOXO1 TUMOR SUPPRESSORS AND THEIR FUNCTION


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# ABSTRACT OF THE PhD THESIS

The FOXO family members (FOXOs) are important tumor suppressors and transcription factors controlling critical intracellular signaling pathways in many different physiological and pathological conditions, such as apoptosis, cell cycle, DNA repair, differentiation, response to oxidative stress, longevity and metabolism and other critical functions, by modulating expression of a wide variety of genes. FOXOs are implicated in regulation of apoptosis by inducing expression of several pro-apoptotic factors acting both on the extrinsic pathway of apoptosis (upregulating TRAIL, FasL, death receptors expression) and on the intrinsic pathway of apoptosis (upregulating Bim, Puma etc). They also regulate proliferation and cell cycle arrest by upregulating p27, p21 and downregulating Cyclin D1.

The inhibition of FOXO tumor suppressors is observed in many malignancies and contributes to tumorigenesis. This suppression can be realized by several mechanisms, including by kinase(such as AKT)-mediated direct phosphorylation and inactivation or by mutations (including formation of fusion mutants). For example, Bcr-Abl, constitutively active tyrosine kinase, mediates inactivation of FOXO3, process critical for the progression of Bcr-Abl positive leukemias. This process can be reversed by the bortezomib-induced indirect activation of FOXO3. Thus, the identification of novel mechanisms that can regulate FOXO tumor suppressors and can be targeted to activate FOXO holds promise in cancer treatment.

The original results presented here bring new critical insights into the regulation and control of FOXO tumor suppressor, in particular of FOXO3. For the first time I identified PLK1 as a novel suppressor of FOXO3 and contributed to the discovery of PP2A as the first phosphatase for FOXO3. While PLK1, a critical kinase for cellular proliferation, can inhibit FOXO3 tumor suppressor and relocate it into the cytoplasm where can be degraded, PP2A phosphatase dephosphorylates FOXO3 at the Akt-dependent phosphorylation sites, activating it and relocating it into the nucleus, where FOXO3 acts as a transcription factor for multiple pro-apoptotic (Bim, Trail) or cell cycle arrest (p27, p21) proteins.

Acting **upstream** of FOXOs, I identified a combined regimen of bortezomib and mitotic inhibitors (known non-specific FOXO3 activators) that efficiently kill Tyrosin-kinase inhibitor sensitive and resistant Bcr-Abl-positive leukemic cells via down-modulation of BCR-ABL.

Moreover, acting **downstream** of FOXOs, we also discovered a novel small molecule which binds and activates caspase 8, designed in the presence of the ligand TRAIL. This small molecule overcomes the resistance to TRAIL-induced apoptosis in multiple cancer cells, including leukemic and prostate cancers cell lines.

Taken together, these results represent not only important advances in our understanding and characterization of FOXO tumor suppressor's regulation and activity (discovery of PLK1 and PP2A regulators of FOXOs) but also provide novel therapeutic strategies with great potential in Bcr-Abl positive leukemia (combined treatment of bortezomib with mitotic inhibitors) and in TRAIL-resistant cancers (a novel small molecule that directly binds caspase 8, sensitizing resistant leukemic and prostate cancer cells to TRAIL-induced caspase 8 activation and TRAIL-induced apoptosis)

## ABBREVIATIONS:

FOXOs (FOXO family members);
NLS (nuclear localization signal);
NES (nuclear export signal);
PLK1 (polo like kinase 1);
c-FLIP (cellular FLICE (FADD-like IL-1β-converting enzyme)-Inhibitory Protein);
TRAIL (tumor necrosis factor-Related Apoptosis-inducing Ligand);
DcR1 (decoy receptor 1); DcR2 (decoy receptor 2);
OPG (osteoprotegerin);
TNF (tumor necrosis factor);
MAPK (mitogen-activated protein kinases);
ERK (extracellular signal-regulated kinase);
NPM (NPM1; nucleophosmin);
Bcl-2 (B-cell lymphoma 2);
NF-kB (nuclear factor-kappa B);
MDM2 (murine double minute 2);
PS (phosphatidil serine);
mTOR (mammalian target of rapamycin);
Bim (Bcl-2 interacting mediator of cell death);
IFN (interferon)
IGF-1 (insulin growth factor 1)
Rb (retinoblastoma protein);
PCAF (CBP associated factor);
Endo G (endonuclease G);
ROS (reactive oxygene species);
GC (Germinal Center);
CML (chronic myelogenous leukemia); AML (acute myelogenous leukemia);
MM (malignant mesothelioma);
SCLC (small cell lung cancer); ALCL (anaplastic large cell lymphoma);

# INTRODUCTION

The FOXO family represents a subclass of the Forkhead transcription factors. According to the last published nomenclature, all forkhead transcription factors have the designed prefix Fox (Forkhead box) and are further divided into subclasses based on their DNA binding domain. FOXO family of transcription factors represent Forkhead Box, Class O. In mammalians, they consist in four members, named FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX) and FOXO6 with many redundant functions, but also with some differences in their regulation and expression in different tissues. The FOXO family is evolutionary conserved, containing a GDSNS sequence in the Forkhead region (DNA binding domain) which is missing in other FOXO family member[1].

The FOXO family members (FOXOs) are tumor suppressors with important functions. FOXOs control critical intracellular signaling pathways in different physiological and pathological conditions[2]. FOXOs are implicated in regulation of apoptosis by inducing expression of several pro-apoptotic factors (Bim, TRAIL, FasL, death receptors expression, Puma etc), in regulation of proliferation (by upregulating p27, p21 and downregulating Cyclin D1) and in other important cellular processes such as DNA repair or cellular differentiation. In my opinion, if I would have to find the transcription factor with the most similarities with FOXOs, I would say that p53 tumor suppressor is the one. P53, similar to FOXOs is a transcription factor and tumor suppressor, transcriptionally induces expression of a number of pro-apoptotic factors and can induce cell cycle arrest by modulation of a similar set of factors (including p21), being also implicated in DNA repair. Moreover, p53 can transcriptionally regulate FOXO3[3] and can directly bind it[4].

FOXOs have a complex posttranslational regulation system. Until know, at least seven different kinases are known to regulate the FOXO family members, by inactivating (AKT, ERK, IKK, SGK etc) or activating (CDK1, MST1, AMPK1) them. We could find the first phosphatase that regulate FOXO3, PP2A[5]. Moreover, FOXOs can also be regulated by acetylation/deacetylation or ubiquitination/deubiquitination, depending on the cellular context.

**Structure of the FOXO family members**

FOXO family members contain a Forkhead domain, which is a DNA binding domain, together with a transactivation domain, which is responsible for FOXO3 functional regulation. In addition, FOXOs have two nuclear localization signals (NLS) and one Nuclear Export Signal (NES). A structural representation of FOXO3 is shown in Figure 1. The main regulatory phosphorylation sites are found in the Forkhead Domain (S253, S318) and N-terminal portion of FOXO3 (Thr32) and are targets of the Akt kinase and PP2A phosphatase, which are the major FOXOs regulatory proteins.

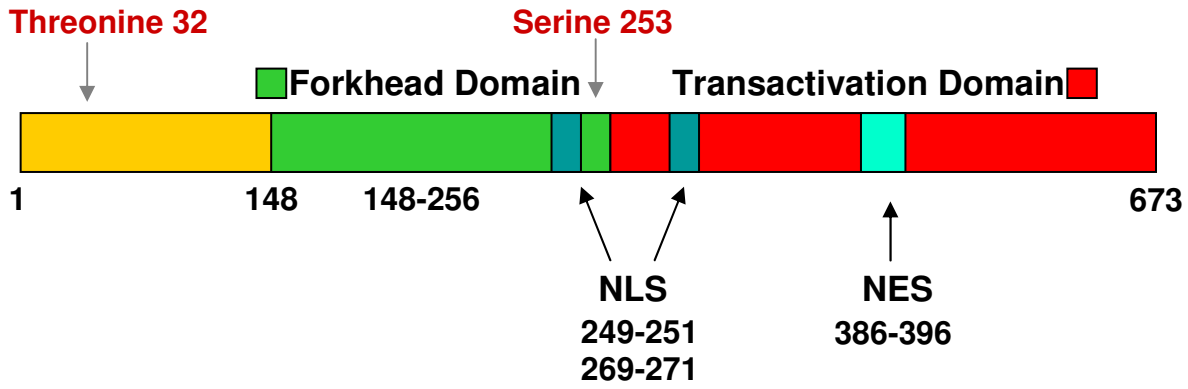


**Figure 1. Schematic representation of FOXO3 structural domains.** Forkhead Domain (DNA binding domain), NLS (Nuclear Localization Signal), NES (Nuclear Export Signal); Thr32 and Ser253 are the most important Akt-phosphorylation sites in FOXO3 (Adapted after[6])

**Functions of the FOXO family members**

FOXO family members are implicated in regulating critical cellular functions, such as apoptosis, cell cycle, DNA repair, differentiation, response to oxidative stress, longevity and metabolism, by modulating expression of a wide variety of genes. A schematic representation of the FOXO functions is presented in Figure 2.

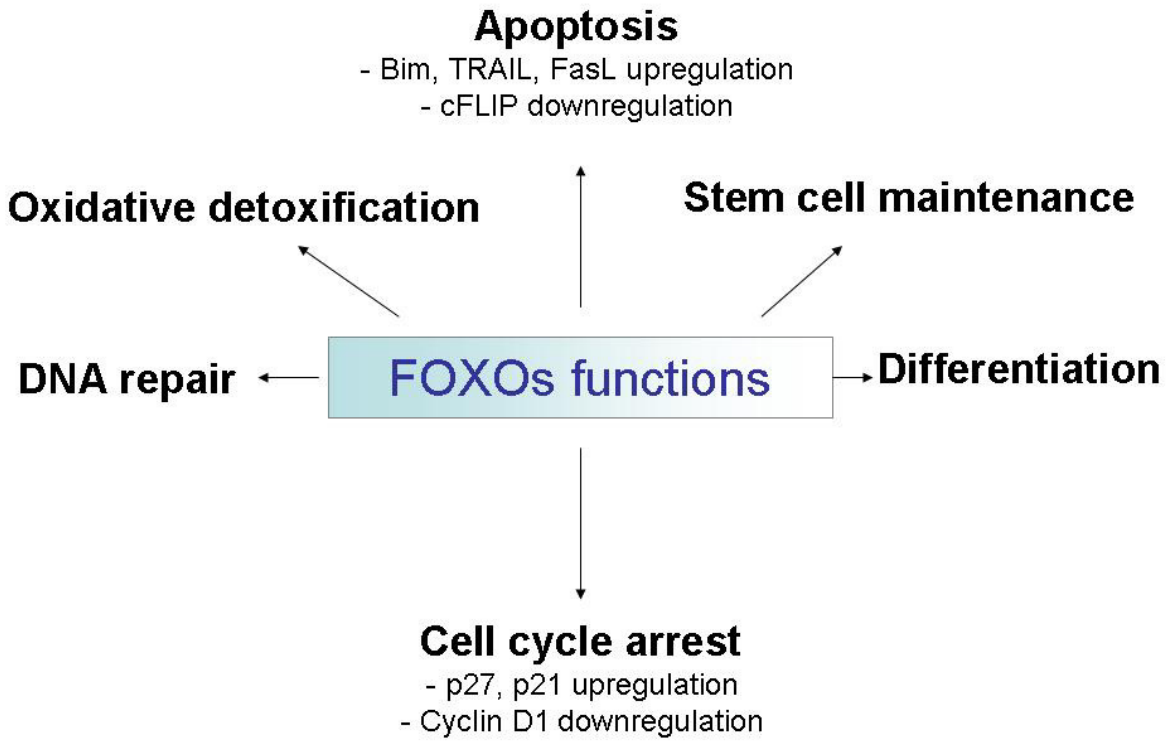


**Figure 2. A schematic representation of FOXO family members function and regulation.** FOXOs have critical function in: apoptosis, cell cycle, stem cell maintenance, differentiation, DNA repair, oxidative detoxification, but not only.[7]

**Regulation of apoptosis by FOXOs**

The main regulatory mechanism of FOXOs is the suppression of these transcription factors by AKT-mediated phosphorylation and exclusion from the nuclei. AKT suppresses FOXOs mediated apoptosis. Initially, three consensus sequences were found in the promoter of FasL, which is a known ligand implicated in inducing apoptosis through the extrinsic pathway of apoptosis[7]. Later, it was shown that FOXO3/FOXO1 can also induce the expression of another death ligand named Apo2L/TRAIL. Furthermore, a number of other pro-apoptotic proteins are upregulated by FOXO3: Bim, Puma and others. Thus, FOXOs can

transcriptionally induce cell death through both the intrinsic (mitochondrial mediated) and extrinsic (death receptors mediated) pathways of apoptosis[7].

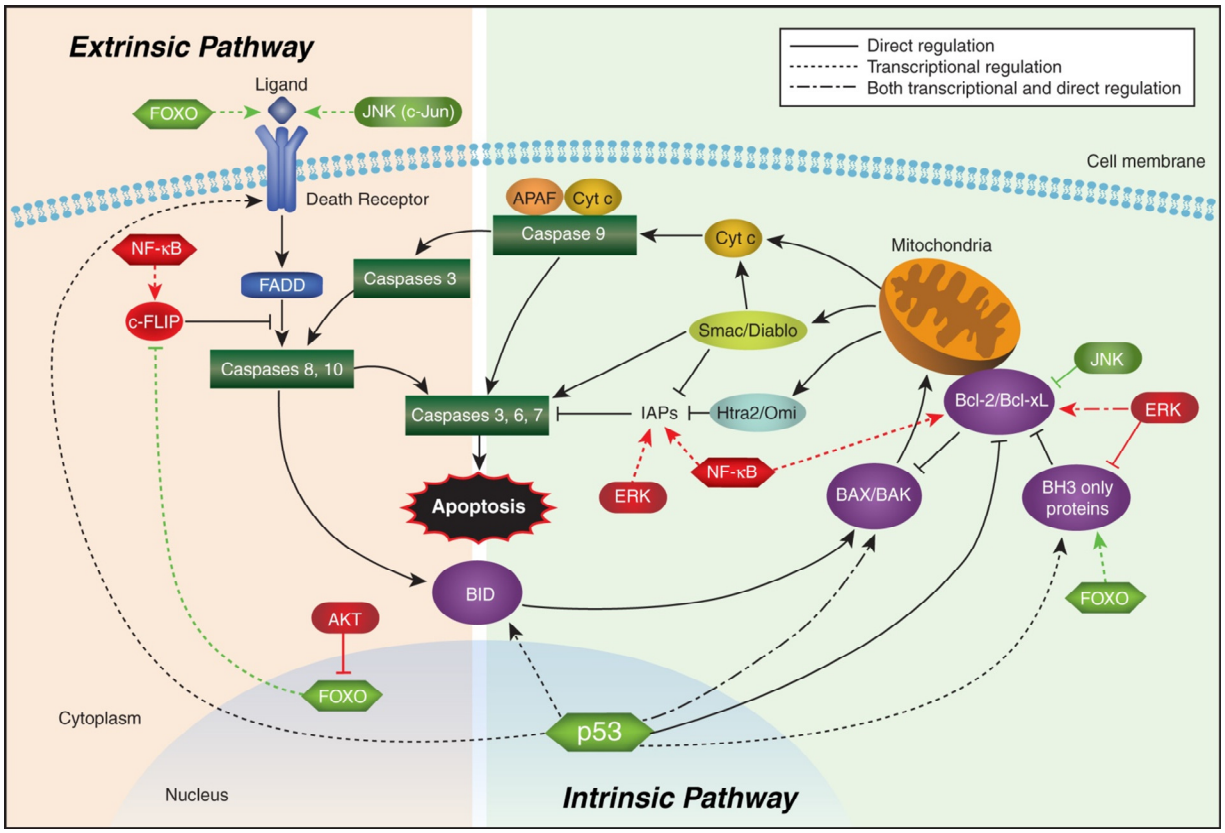


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**Figure 3. FOXOs regulation of the intrinsic and extrinsic pathway of apoptosis.**

FOXOs induce the expression of death ligands and inhibit c-FLIP expression, which stimulates induction of apoptosis on the extrinsic pathway of apoptosis, while FOXOs induced expression of the BH3 only protein Bim induces apoptosis on the intrinsic pathway of apoptosis (unpublished figure).

**Regulation of cell cycle and proliferation by FOXOs**

While FOXOs activity can induce apoptosis when overexpressed in many cell types, in some cell types it can induces at some concentrations cell cycle arrest in G1. This process is transcriptionally FOXOs mediated by upregulation of the cell cycle inhibitor p27. Moreover, in other two reports, FOXOs were shown to upregulated the p21 cell cycle inhibitory protein and the Retinoblastoma protein (RB) related protein named p130, another suppressor of G1/S transition. FOXOs can also downregulate Cyclin D1 and Cyclin D2.

Surprisingly, while expression of FOXOs induces cell cycle arrest in G1, some FOXO3 expression is needed in M phase of M-G1 phase transition and for stimulating the expression of critical cell cycle kinase PLK1 and Cyclin B1[7].

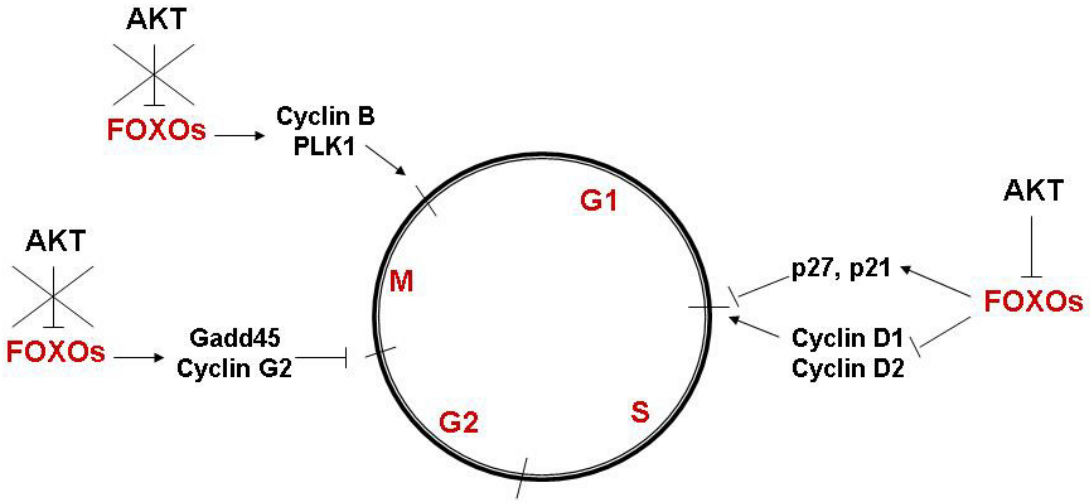



**Figure 4. FOXO3 functions during cell cycle**

Adapted/modified after Huang H, Tindall DJ. J Cell Sci. 2007[7]

**Regulation and targeting of apoptosis by modulating proteasomal degradation**

Numerous proteins critical for the regulation of apoptosis, proliferation, differentiation or other important cellular functions have been revealed to undergo processing by a proteasome degradation pathway[8-13]. Between them we can identify many tumor suppressors, oncogenes, transcription factors, metabolic enzymes and cell cycle regulators. The expression levels of many of these key proteins are critically regulated by ubiquitination, marking and targeting them for degradation through the proteasome pathway[14,15]. The 26S proteasome is an ATP-dependent multi-subunit protease complex found in both the cytoplasm and nuclei of eukaryotic cells[15].

Apoptotic pathways, similar to other cellular signaling pathways have complex control mechanisms. One of this regulatory mechanism is represented by poly-ubiquitination and rapidly degradation by proteasome of critical proteins, which are implicated in the response to either pro- or anti-apoptotic stimuli. This assures a right execution or inhibition of apoptosis[16,17]. Interestingly, proteasome-mediated degradation plays an important role in the inactivation of growth inhibitors/tumor suppressors in many tumors. This results in the activation of several distinct growth stimulating proteins and inhibition of apoptosis, therefore underscoring the degradation induced by the proteasome as an attractive target in cancer therapy[18].

Bcr-Abl expression not only regulates phosphorylation of the pro-apoptotic FOXO3 transcription factor[1,19,20], but also promotes a significant reduction in the total FOXO3 protein levels in a proteasome-dependent manner (unpublished results). Since FOXO3 induces activation of apoptosis in hematopoietic cells and because Bcr-Abl-induced proteasomal degradation of FOXO3 will promote apoptotic evasion of Bcr-Abl-expressing hematopoietic cells and subsequent survival and tumorigenesis of these cells, *one strategy can be to target FOXO3 degradation in order to re-established the critical levels of active FOXO3.* These levels are important for maintaining normal cellular homeostasis.

**Bortezomib, a selective proteasome inhibitor, as a novel treatment of Bcr-Abl positive leukemias**

It is known that bortezomib (velcade, PS-341), a selective proteasome inhibitor (approved by European Medicines Agency & US Food and Drug Administration (FDA) for

the treatment of multiple myeloma and mantle cell lymphoma) efficiently inhibits survival and induces apoptosis in imatinib-resistant Bcr-Abl cells[7]. Bortezomib significantly reduces the signs of CML-like disease in Bcr-Abl transduced mice[7]. Moreover, it was also reported that bortezomib treatment caused remission in a patient with Bcr - Abl positive Acute Lymphoblastic Leukemia (ALL), refractory to standard therapies[7]. An excellent response with a complete remission, maintained for more than for years since the patient's initial diagnosis and beginning of the therapy, was observed[7]. Based on these results, at least six different clinical trials have been performed or are currently performed, using bortezomib alone or in combination with other drugs for the treatment of CML and/or Ph+ALL[7]. Thus, *bortezomib is a promising treatment in Bcr-Abl-positive leukemias.*

*Noteworthy, bortezomib was previously reported to re-activate FOXO family of tumor suppressors in vitro and in vivo, resulting in cell death of both Imatinib sensitive and resistant Bcr-Abl positive cells.* Moreover, bortezomib can re-sensitize the Imatinib-resistant and -sensitive Bcr-Abl positive cells to Imatinib[7]. Treatment with bortezomib can overcome the Bcr-Abl - induced inhibition of FOXO proteins, inducing the expression of FOXO-dependent pro-apoptotic targets such as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and Bim. Apoptosis induction is at least in part due to re-activation of FOXO tumor suppressors[7]. These results highlight the potential importance of the proteasome inhibitors in cancer therapy.

**Apoptosis process regulation and general characteristics**

Apoptosis is a well controlled cell suicide program important in normal development and tissue homeostasis. Apoptosis is a physiologic process that eliminates unwanted and especially unnecessary cells[19]. This is a conserved type of programmed cell death that is essential for the removal of cells infected with viruses, cells with DNA mutations and damage, and cancer cells. The right apoptotic signaling and control of apoptosis is needed to maintain the balance between cell death and cell survival. Evasion from apoptosis had been shown to represent an important hallmark of cancer[20].

Caspases are a family of proteases (cysteinyl, aspartate-specific proteases) and are considered the main effectors of apoptosis. Importantly, caspases are expressed as inactive zymogens that are activated when cleaved. In response to stimulation of the death receptors,

the initiator caspases are cleaved and activated, inducing the activation of a second class of caspases, named effector caspases[21]. Effector caspases dismantle the cell by cleaving key proteins and other cellular components, such as PARP (poly(ADP-ribose) polymerase), and inducing biochemical and morphological changes specific to apoptotic cells. These alterations that are characteristic to apoptosis include: cellular membrane blebbing, movement of the PS (phosphatidylserine) from the internal side of the cellular membrane on the external, chromatin condensation, and DNA fragmentation[22]. The apoptosis process is finalized with the formation of apoptotic bodies, containing parts of cytoplasm, nuclei and organelles. that are recognized and eliminated by neighboring cells or phagocytes by phagocytosis[22,23]. Activation of the effector caspases (such as 3, 6 and 7), and the subsequent cell death induction, can be triggered by two pathways: extrinsic and intrinsic pathways of apoptosis. While extrinsic pathway of apoptosis is mediated by death receptors, the intrinsic pathway of apoptosis is mediated by mitochondria inner membrane depolarization and permeabilization[21]. (Figure 5).

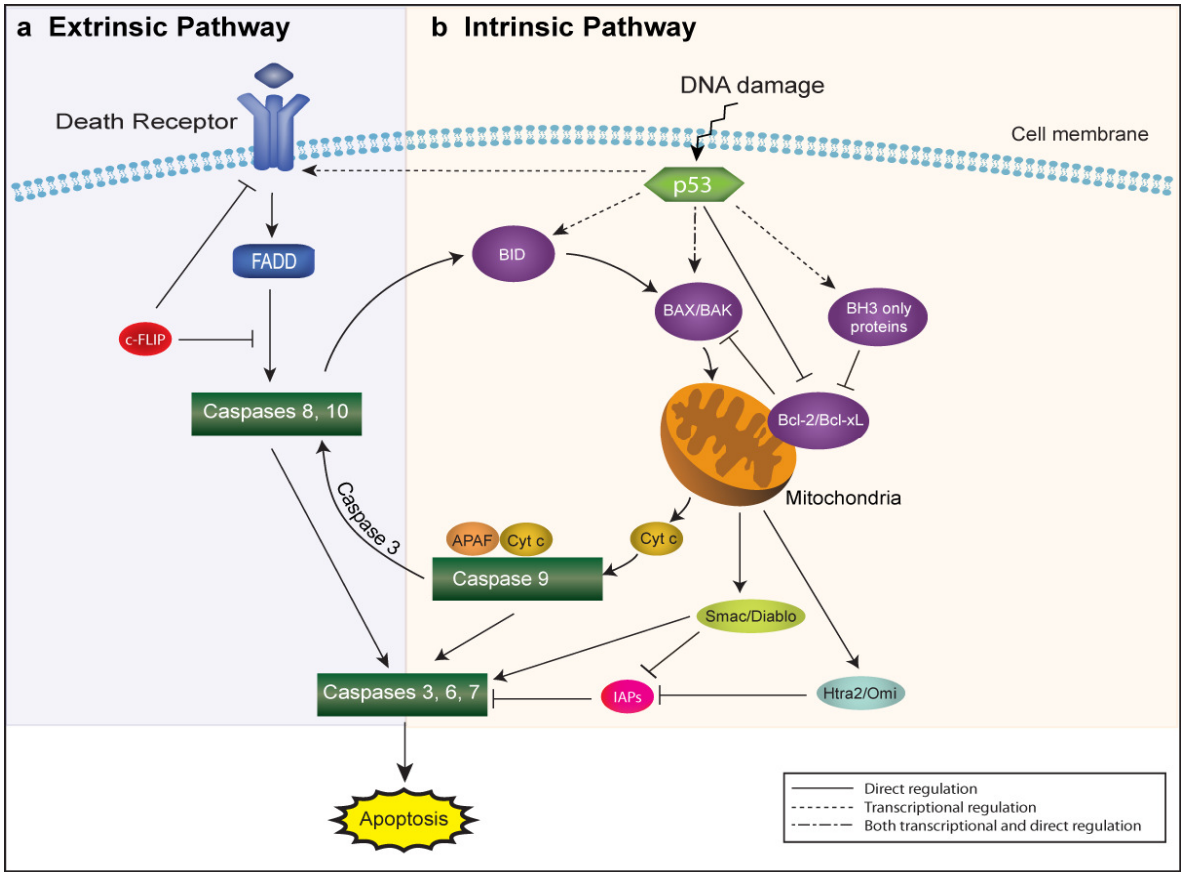


Figure 5 | The **extrinsic and intrinsic apoptotic pathways are presented.** Apoptosis is mediated by two major apoptotic pathways, the extrinsic (death receptor-dependent) pathway or the intrinsic (mitochondria-dependent) pathway **a** | Stimulation of the cell surface death receptors by their respective ligands activates the extrinsic pathway, leading to the activation of the initiator caspases-8 and -10, which in turn activates the effector caspases-3, -6 and -7 that execute various protein cleavage events to ensure cell death. **b** | The intrinsic pathway is activated in response to diverse apoptotic stimuli, including DNA damage, which relay the death signal to the mitochondria, resulting in mitochondrial outer membrane permeabilization (MOMP) and the subsequent release of apoptogenic factors from the mitochondria (cytochrome c, Omi/Htr2A, Smac/Diablo). Upon being expelled into the cytoplasm, cytochrome c (cyt c) forms a complex with APAF and caspase 9 to promote the activation of the initiator caspase 9 and the subsequent activation of the downstream executioner caspases-3, -6 and -7. The extrinsic and intrinsic apoptotic pathways are linked by the caspase 8-induced cleavage of Bid, resulting in the translocation of the C-terminal Bid fragment to the mitochondria, where it promotes MOMP and the release of apoptogenic factors. The cyt c-dependent activation of caspase 9 can amplify the induction of apoptosis by caspase 8 through caspase 3 activation, resulting in a positive feedback loop *(unpublished figure)*

# MATERIALS and METHODS (selection)

Due to the high number of techniques employed, a selection of them is presented here. For a detailed description of the methods used for each individual results chapter, please consult the full PhD Thesis.

**Cell lines and cell culture reagents**

All cell lines were purchased from ATCC. The K562 leukemic cell line and the PC3 and PC3-TR prostate cancer cell lines were grown in RPMI 1640 medium (Mediatech Inc., VA, USA, Cat. # 10-040-CV) supplemented with 10% fetal bovine serum (FBS, HyClone, Cat. # SH30088.03), L-glutamine (Mediatech, VA, USA, Cat. # 25-005-CI) and Penicillin/Streptomycin (Mediatech Inc., VA, USA, Cat. # 30-001-CI). The HCT115 and HCT116 colon cancer cell lines were grown in McCoy's 5A medium (Mediatech Inc., Cat. # 10-050-CV) supplemented with 10% FBS, L-glutamine and Penicillin/Streptomycin. The MCF7 and MDA231 breast cancer cell lines and the ASPC1 and MIAPACA pancreatic cancer cell lines were grown in DMEM medium (Mediatech, VA, USA, Cat. # 10-013-CV) supplemented with 10% FBS, L-glutamine and Penicillin/Streptomycin. Similar, HeLa and 293T cells were grown in supplemented DMEM medium. The K562 imatinib-resistant cell lines (K562-R) and LAMA84 imatinib-resistant cell lines (LAMA-R) were generated in our laboratory, by incubation of the K562 and LAMA84 cells, respectively, with increasing concentrations of imatinib mesylate for 3-4 weeks.

**Western Blotting**

SDS-PAGE and the immunoblotting were performed as previously described[5,8]. Whole cell lysates (30 μg) in reducing Laemmli's SDS Sample Buffer (Boston Bioproducts, MA, USA, Cat # BP-110R) were resolved by 10% SDS-PAGE, and transferred to 0.45 μm Immobilon-P membranes (Millipore, MA, USA Cat # IPVH00010). The membranes were then blocked for 30 min at room temperature in blocking buffer (5 % w/v non-fat dry milk/Tris-buffered saline-Tween-20) (TBST: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05 % (v/v) Tween-20). Rabbit/mouse primary antibodies were diluted in 5% (w/v) blocking buffer then incubated with membranes overnight at 4°C, or for 2 h at room temperature. Anti-c-FLIP antibodies 1, 5 & 6 were used at dilution of 1:1,000, antibody 2 at dilution of 1:333-1:1,000 and antibodies 3 and 4 at concentration of 1 μg/ml, as recommended by the manufacturer.

Membranes were then washed three times with TBST, incubated for 1 h at room temperature with either HRP-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibodies (sc-2055 and sc-2054, respectively, Santa Cruz Biotechnology, CA, USA) diluted 1:3,500 in blocking buffer, and then washed again with TBST prior to visualization using a chemiluminescent substrate (Pierce ECL Western Blotting Substrate, Super Signal West Pico or Super Signal West Femto, Pierce - Thermo Scientific, IL, USA). β-Actin was detected by using a 1:20,000 dilution for both primary and secondary antibodies.

**Immunoprecipitation/Co-immunoprecipitation and Tandem affinity purification (TAP)**

Briefly, HeLa cells stably expressing FOXO3 fused to tandem FLAG and HA epitopes were lysed in EBC buffer as previously described[5]. Cell lysates were first immunoprecipitated with anti-FLAG (M2) affinity gel (Sigma) for 2h at 4C. FLAG immunocomplexes were washed three times with EBC lysis buffer and FLAG-HA-FOXO3 was eluted with 150ng/µl 3X FLAG peptide (Sigma). FLAG peptide eluates were then immunoprecipitated with anti-HA (HA7) agarose affinity resin (Sigma) for 2h at 4C, washed three times with EBC buffer, and eluted with 1µg/µl HA peptide (Sigma). Lysate were then fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then silver stained. The marked protein bands were excised from the silver stained gel and analyzed by microcapillary LC/MS/MS techniques (by the Taplin Mass Spectrometry Core Facility Harvard Medical School), using a LTQ FT Ultra Hybrid Mass Spectrometer (Thermo Electron).

**Live-Cell Microscopy and image analysis of single cells**

Time-lapse microscopy movies were recorded with a Deltavision fluorescence microscope equipped with an environmental chamber (Olympus, Applied Precision) at 10x magnification with frames every 3 minutes for 24 hours. Cells grown in 8-well chambered cover glass slides (Nunc) were shifted into phenol red-free medium (Invitrogen) supplemented with 10% fetal bovine serum and L-glutamine for imaging. For FRET signal analysis, ratio of background-subtracted CFP and YFP images were created by using ImageJ and custom plug-ins. Signals were normalized by subtracting the minimum value across all time points from each single-cell time course. IMS-RP release in cell cytoplasm was analyzed in ImageJ.

# RESULTS

**Combination of bortezomib and mitotic inhibitors efficiently kills Tyrosin-kinase inhibitor sensitive and resistant Bcr-Abl-positive leukemic cells via down-modulation of BCR-ABL (selected figures are presented)**

The inactivation of FOXO tumor suppressors is observed in many malignancies and contributes to tumorigenesis. This suppression can be realized by several mechanisms, including by kinase (such as AKT)-mediated direct phosphorylation and inactivation or by mutations (including formation of fusion mutants). Bcr-Abl, a constitutively active tyrosine kinase, mediates inactivation of FOXO3, process critical for the progression of Bcr-Abl positive leukemias. This process can be reversed by the bortezomib-induced indirect activation of FOXO3[8].

Emergence of resistance to Tyrosine-Kinase Inhibitors (TKIs), such as imatinib, dasatinib and nilotinib, in Chronic Myelogenous Leukemia (CML) demands new therapeutic treatments. This chapter describes the development of a novel strategy of efficiently killing Tyrosin-kinase inhibitor sensitive and resistant Bcr-Abl-positive leukemic cells via down-modulation of BCR-ABL, by using a combined treatment with Bortezomib and mitotic inhibitors. This strategy is especially important in the Bcr-Abl positive leukemias resistant to current treatments, such as Tyrosine Kinase Inhibitors[8].

The results demonstrate that bortezomib in combination with the mitotic inhibitor paclitaxel, efficiently kill TKIs-resistant and -sensitive Bcr-Abl-positive leukemic cells. Moreover, bortezomib, in combination with either paclitaxel, or BI 2536, another mitotic inhibitor that inhibits PLK1, induces a marked downregulation of protein levels of total and phosphorylated Bcr-Abl, thus downregulating the critical Bcr-Abl downstream signaling pathways and caspase activation. Similarly, bortezomib, in combination with other mitotic inhibitors (vincristine and docetaxel), is able to decrease Bcr-Abl activity and increase caspase activation.

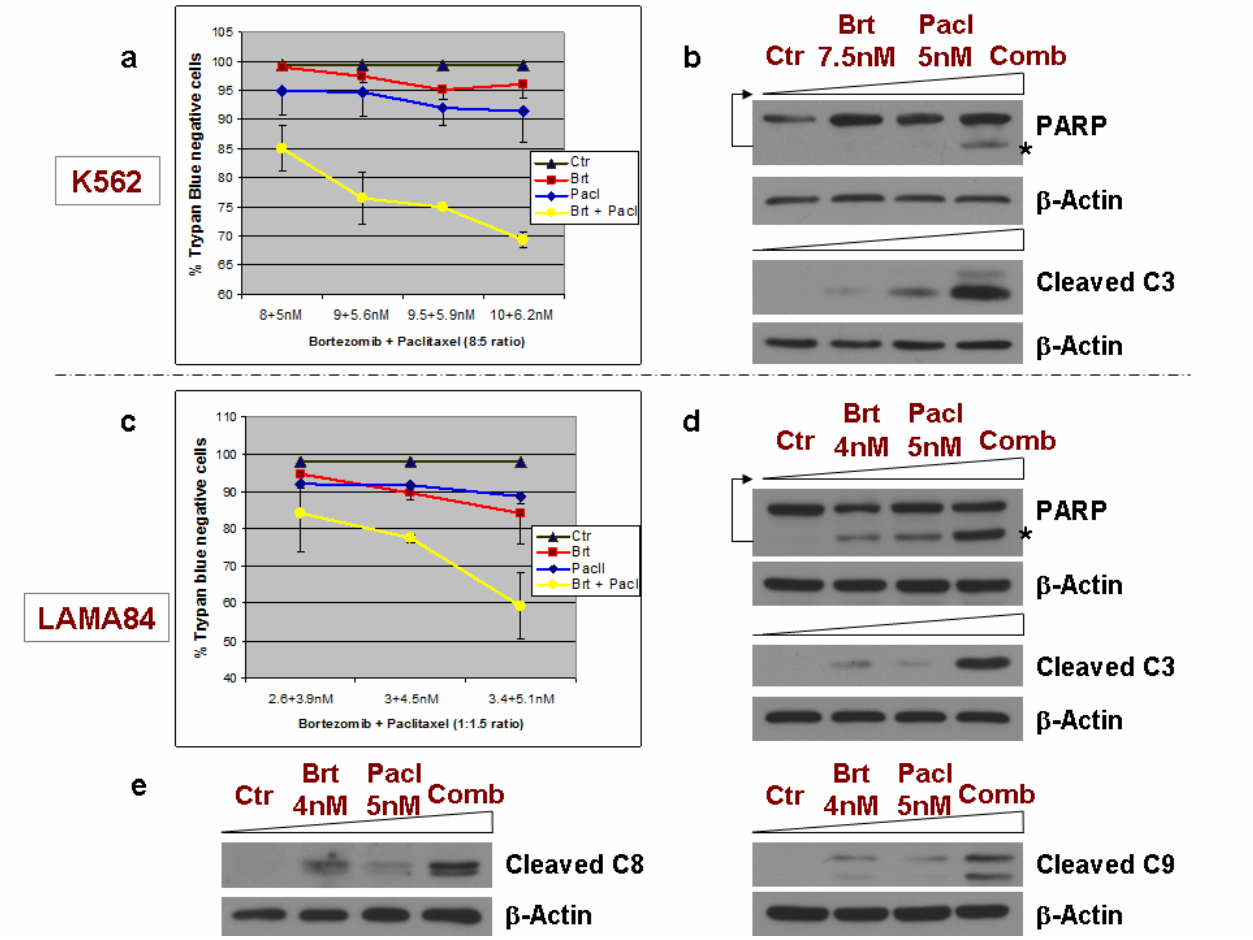



**Figure 6. Combined treatment of bortezomib and paclitaxel efficiently activates caspases and induces cell death in human leukemic Bcr-Abl-positive K562 and LAMA84 cell lines**

(**a**) K562 leukemic cells were exposed to bortezomib (8nM, 9nM, 9.5nM and 10nM) with or without paclitaxel (5nM, 5.6nM, 5.9nM, 6.2nM) for 48h. The percentage of cell death was measured with an automated Trypan Blue exclusion method. The combination significantly increased the number of Trypan Blue-positive cells, compared with each drug used alone. The results are a mean of two separate experiments. (**b**) K562 leukemic cells were treated with 7.5nM bortezomib and 5nM paclitaxel for 48h, followed by detection of the cleaved fragments of caspase 3, and of PARP cleavage. The combined regimen induced significant cleavage of caspase 3 and PARP, implying caspase activation. (**c**) LAMA84 leukemic cells were exposed to bortezomib (2.6nM, 3nM and 3.4nM) with or without paclitaxel (3.9nM, 4.5nM and 5.1nM), at a fixed ratio of 1:1.5, for 48h. The percentage of cell death was measured with an automated Trypan Blue exclusion method. The combination significantly increased the number of Trypan Blue-positive cells, compared with each drug used alone. The results represent a mean of three separate experiments. (**d**) LAMA84 leukemic cells were treated with 4nM bortezomib and 5nM paclitaxel for 48h, followed by detection of the cleaved fragments of caspase 3, caspase 8, caspase 9 and of PARP cleavage. The combined regimen significantly enhanced the cleavage of caspases 3, 8, 9 and PARP, suggesting caspase activation. At least 3 separate experiments were performed in each case, except where stated otherwise. β–Actin was used as a loading control.

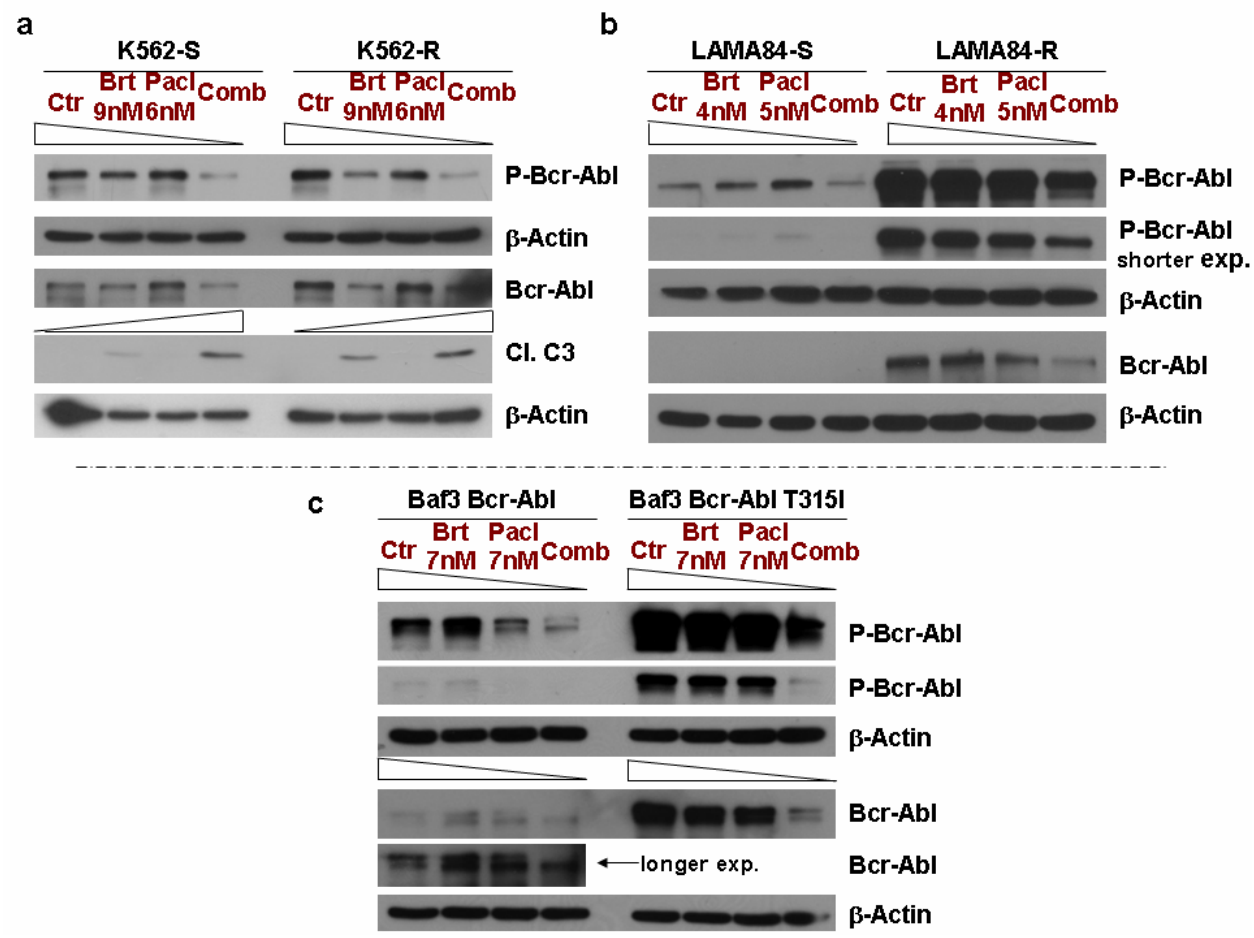



**Figure 7. Bortezomib/paclitaxel combination induces downregulation of the total levels and phosphorylation of Bcr-Abl in TKIs-resistant K562-R, LAMA84-R and Baf3 Bcr-Abl T315I, displaying increased Bcr-Abl expression/activity and/or T315I mutation**. (**a**) K562 (K562-S) and K562-R leukemic cells were treated with 9nM bortezomib and 6nM paclitaxel for 48h, followed by detection of the total levels and phosphorylation of Bcr-Abl. The combined regimen significantly downregulates the total levels and phosphorylation of Bcr-Abl in both cell lines, and this effect correlates with a significant increase in caspase 3 cleavage. (**b**) LAMA84 (LAMA84-S) and LAMA84-R leukemic cells were treated with 4nM bortezomib and 5nM paclitaxel for 48h, followed by detection of the total levels and phosphorylation of Bcr-Abl. The combined regimen downregulates the total levels and phosphorylation of Bcr-Abl in LAMA84-R and phosphorylation of Bcr-Abl in LAMA84-S. (**c**) Baf3 Bcr-Abl and Baf3 Bcr-Abl T315I cell lines were treated with 7nM bortezomib and 7nM paclitaxel for 48h, followed by detection of the total levels and phosphorylation of Bcr-Abl. The combined regimen downregulates the total levels and phosphorylation of Bcr-Abl in both cell lines. β-Actin was used as a loading control. One representative experiment from several separate experiments is shown.

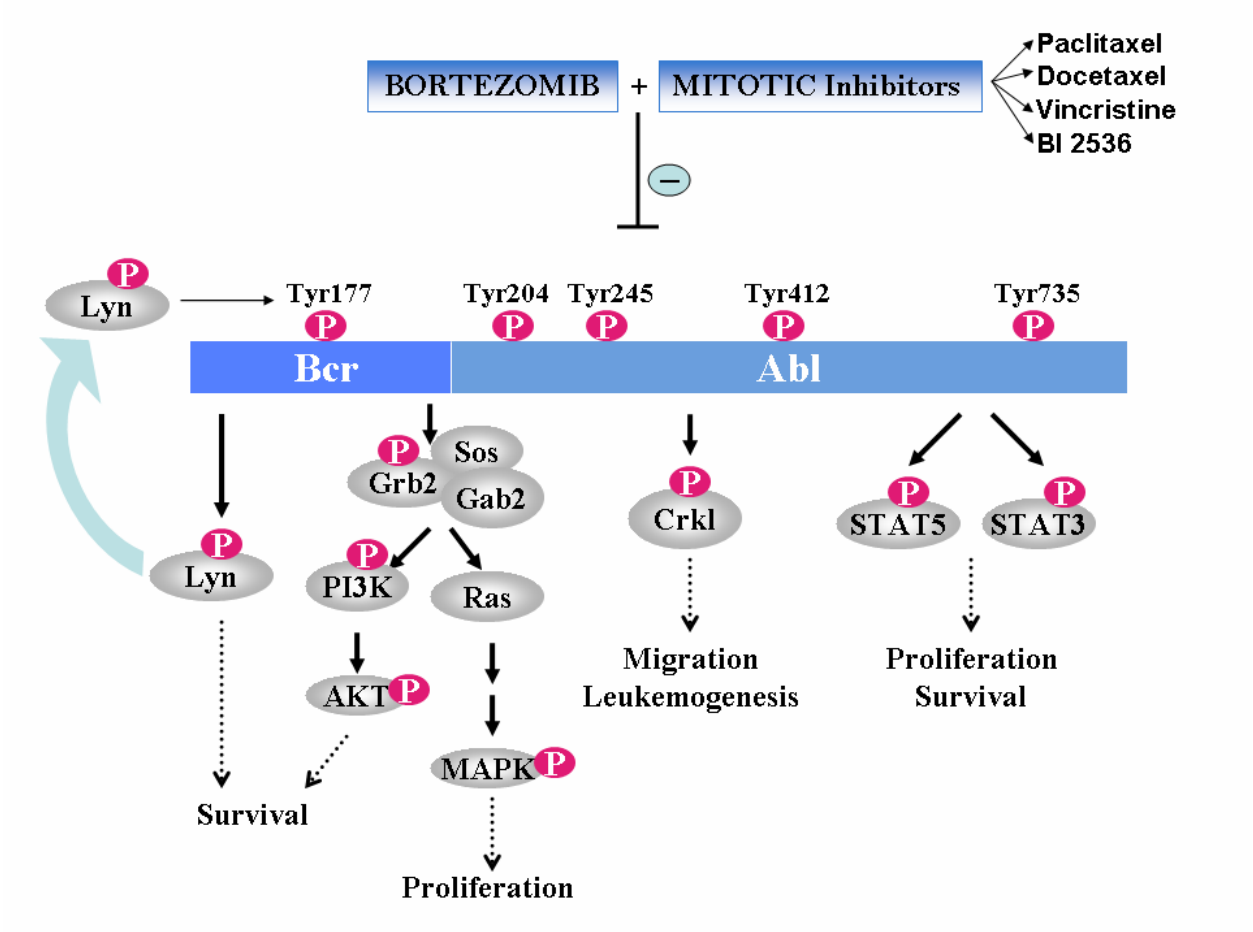
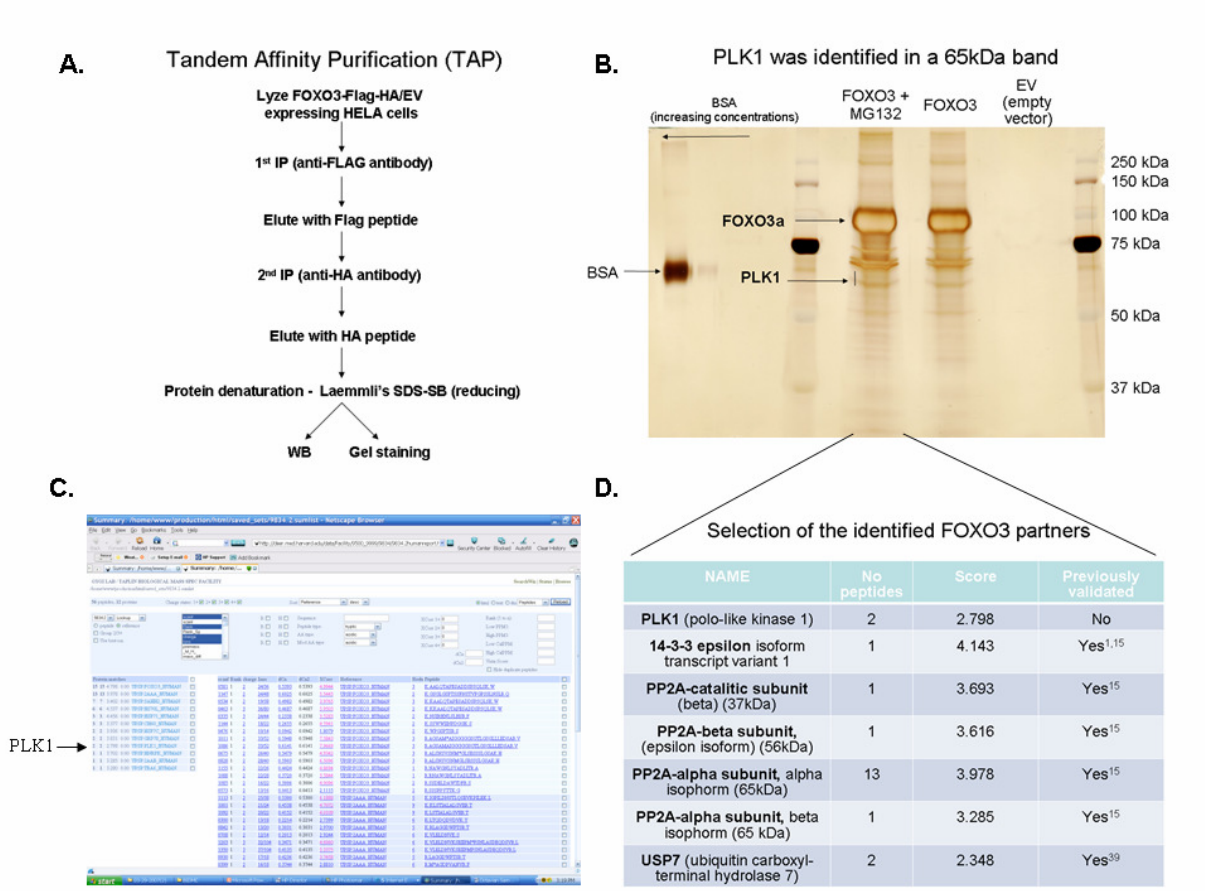



**Figure 8. Bortezomib in combination with mitotic inhibitors as a novel strategy for CML: a model of the combined treatments-induced cell death[24-32].** Bcr-Abl is phosphorylated at multiple phosphorylation sites, resulting in binding/phosphorylation of downstream Bcr-Abl mediators. As an example, Tyr177 phosphorylation mediates Bcr-Abl downstream signaling by inducing the formation of a Lyn-Gab2-Bcr-Abl complex, and is required for Bcr-Abl-induced leukemia[32,33]. This binding results in Lyn activation by phosphorylation[34]. Lyn kinase further regulates survival and responsiveness of CML cells to inhibition of Bcr-Abl kinase[25]. Interestingly, Lyn can also phosphorylate Tyr177 in Bcr-Abl[32], resulting in a potential feedback mechanism. The Gab2-Bcr-Abl complex is mediated by Grb2 and results in activation of downstream proliferative/survival pathways, such as Ras-ERK and PI3K-Akt. Gab proteins couple growth factor and cytokine receptors to downstream proteins, resulting in activation of the downstream pathways Ras-ERK, PI3K-Akt and JAK/STAT pathways[35]. Bcr-Abl phosphorylates and activates STAT3 and STAT5 transcription factors inducing survival and proliferation. Constitutive activation of STAT3/STAT5 is critical for the maintenance of chronic myeloid leukemia[26,36]. Bcr-Abl also binds the C-terminal Proline-rich region of the adaptor protein CrkL[36]. Bcr-Abl phosphorylates CrkL, an event needed for Bcr-Abl-induced leukemia. CrkL can enhance cell migration and Bcr-Abl-mediated leukemogenesis[24,38-40].Our results show that bortezomib in combination with several mitotic inhibitors, known to suppress mitosis through different mechanisms, is able to downregulate total levels and phosphorylation of Bcr-Abl at the Tyr 177 site and to inactivate the Bcr-Abl downstream pathways, mediated by Lyn, CrkL or STAT3/STAT5. These effects are associated with caspase-mediated cell death.

**A Tandem Affinity Purification screening identifies novel FOXO3 binding partners: PLK1 is a negative regulator of FOXO tumor suppressors (selected figures are presented)**

In order to identify novel regulators of FOXO3 localization and activity, we have employed a proteomics screening strategy. Using HeLa cancer cell line and a Tandem Affinity Purification followed by Mass Spectrometry analysis, I identified several proteins as novel binding partners of FOXO3. Noteworthy, Polo Like Kinase 1 (PLK1) was one of the identified FOXO3 binding partner. PLK1 is a member of the Polo-Like kinases family, comprising in humans PLK1, PLK2, PLK3 and PLK3, a conserved family of kinases, critical in regulation of cell cycle/proliferation and in DNA damage-induced checkpoints[41]. PLK1, the most important family member, plays a critical role during mitosis and in maintenance of genomic stability. PLK1 is overexpressed in a wide range of malignancies (including but not limited to melanoma, non-small lung cancer, gastro-instestinal cancers and prostate cancer). Interestingly, PLK1 is associated with an increased risk for metastasis[41-44]. Several PLK1 inhibitors were developed PLK1 suppression with small molecules is analyzed in clinical trials for the treatment of Acute Myelocytic Leukemia (AML) and other malignancies[8,45-48].

Our experimental results demonstrate that FOXO3 and PLK1 exist in a molecular complex through most of the phases of the cell cycle, with a higher occurrence in the G2-M phases of the cell cycle. PLK1 induces translocation of FOXO3 from nucleus to the cytoplasm and suppresses FOXO3 activity, measured by the decrease in the pro-apoptotic Bim protein levels and decrease in the cell cycle inhibitor protein p27. Furthermore, PLK1 can directly phosphorylate FOXO3 in an *in vitro* kinase assay. These results present the discovery of a novel kinase, PLK1, which binds and suppresses FOXO3 localization and activity.




| NAME | No peptides | Score | Previously validated |
|---|---|---|---|
| **PLK1** (polo-like kinase 1) | 2 | 2.798 | No |
| **14-3-3 epsilon** isoform transcript variant 1 | 1 | 4.143 | Yes[1,15] |
| **PP2A-catalitic subunit** (beta) (37kDa) | 1 | 3.693 | Yes[15] |
| **PP2A-beta subunit,** (epsilon isoform) (56kDa) | 1 | 3.616 | Yes[15] |
| **PP2A-alpha subunit,** alpha isophorm (65kDa) | 13 | 3.978 | Yes[15] |
| **PP2A-alpha subunit,** beta isophorm (65 kDa) | 1 | 3.285 | Yes[15] |
| **USP7** (ubiquitin carboxyl-terminal hydrolase 7) | 2 | 2.348 | Yes[39] |

**Figure 9. Novel FOXO3 binding partners discovered by using a TAP-MS strategy; A.** Tandem Affinity Purification (TAP) followed by protein identification by Mass Spectrometry (MS) method was employed to identify novel FOXO3 binding partners. Lysate from Hela cells transfected with either FOXO3-Flag-HA or EV-Flag-HA were immunoprecipitated with an anti-FLAG antibody, followed by a second purification by using an anti-HA antibody. Denatured proteins (reducing Laemmli's SDS-SB) were separated by SDS-PAGE. **B.** Proteins were stained with Silver stain. The bands corresponding to FOXO3 and PLK1 proteins identified by MS are presented. BSA (Bovine Serum Albumin) was used as a marker for protein quantity. **C.** Proteins identified by MS in the 60-65 kDa band presented in (B). PLK1 was one of the identified proteins (the arrow indicates PLK1). **D.** Most important/promising proteins identified after analyzing multiple bands by MS, covering 25-200 kDa gel range, are presented. EV – empty vector; MG132 was used to enrich for potential E3 ligases.

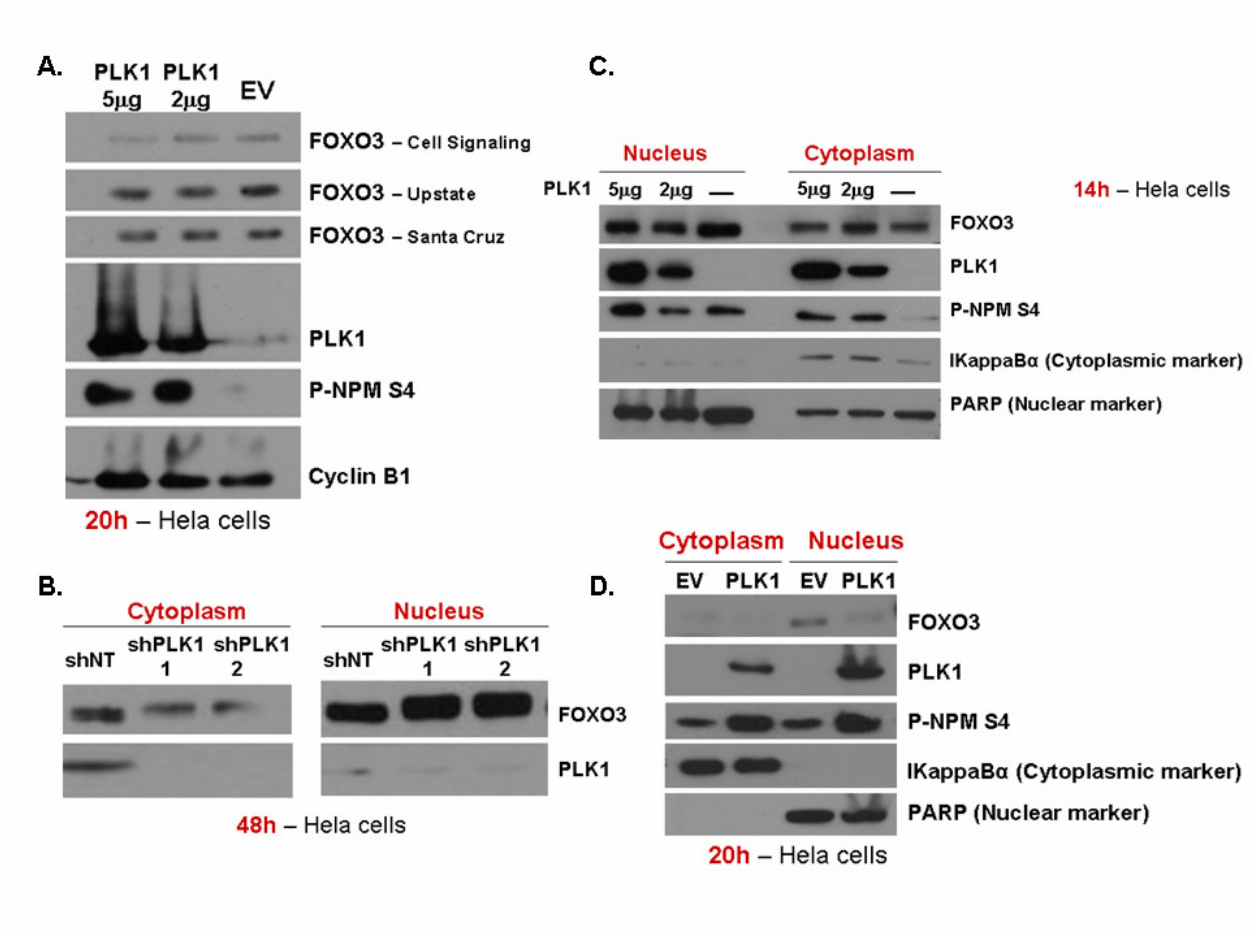



**Figure 10. PLK1 regulates FOXO3 nuclear localization and total levels; A.** Hela cells were transfected with either EV-FLAG or increasing concentrations (2ug, 5ug) of PLK1-FLAG. FOXO3 total levels are downregulated by PLK1 overexpression after 20h from transfection. Cyclin B1 is used as an internal loading control. **B.** Hela cells were transfected with either two different shRNA for PLK1 or a non-targeting shRNA (shNT) for 48h. PLK1 knockdown induces FOXO3 relocalization into the nucleus. Nuclei and cytoplasm were isolated and FOXO3 levels were evaluated in each fraction. **C. & D.** Hela cells were transfected with either EV-FLAG or PLK1-FLAG. PLK1 expression induces a decrease of the nuclear FOXO3 fraction at both 14h (**C.**) and 20h (**D.**), suggesting a decrease in FOXO3 activity. PARP is used as a nuclear marker, while IkappaBα is used as a cytoplasmic marker; PLK1 activity is determined by measuring the phosphorylation of NPM at Ser4 (site phosphorylated by PLK1)[43]

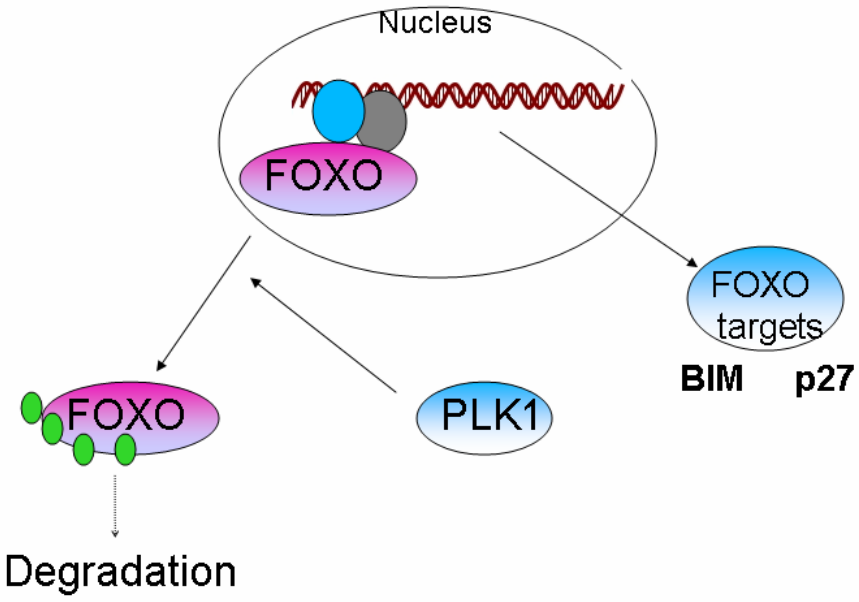



**Figure 11. Model of how PLK1 controls FOXO3 localization, levels and activity.**
PLK1 forms a complex with FOXO3 and other FOXO family members and suppresses them. The binding takes places during most phases of the cell cycle, being better observed during the G2-M phases. PLK1 induces the exclusion of FOXO3 from the nucleus, resulting in a decrease in the expression of FOXO3-dependent targets Bim and p27, while lack of PLK1 can induce the opposite effect, resulting in FOXO3 accumulation into the nuclei. Moreover, PLK1 is able at least *in vitro* to phosphorylate FOXO3 at multiple sites, suggesting that PLK1 may be a potential FOXO3 kinase. Our results highlight the importance of a new regulatory mechanism of FOXO tumor suppressors by the PLK1 proto-oncogene;

**The status quo of poor antibody characterization is a challenge in biomedical research: c-FLIP (an important FOXO3 target) endogenous protein detection with six currently used antibodies reveals high non-specificity ((selected figures are presented)**

Low reproducibility of the published preclinical research results has been recently highlighted in several articles including a commentary by Begley and Ellis in March 2012 issue of Nature[49]. This problem contributes significantly to the low success rate of translating findings derived from preclinical research into effective treatments. Methods based on antibodies are amongst the most widely utilized techniques in biomedical research and as such the specificity and accuracy of antibodies employed is pivotal. I sought to determine if the status quo of antibody characterization is sufficient to ensure reliable accuracy of findings in peer-reviewed published reports. To explore this issue, I chose to test antibodies generated against c-FLIP, a major factor of resistance to apoptosis induced by many cytokine-/death ligand-based therapeutics that is a potential theranostic biomarker. FOXO3 is a transcription factor that induces the downregulation of c-FLIP[50-52].

Several sources of c-FLIP antibodies that have been used in prior reports were rigorously tested. Surprisingly, we discovered a wide divergence in the immune recognition properties amongst these antibodies. Moreover, several of these antibodies failed to detect the endogenous c-FLIP protein. Our results underscore that current practices of antibody characterization are insufficient and provide an often over-looked source of data inaccuracy.

We strongly encourage higher standards for validation of all antibodies that require genetic knockdowns and/or knockouts to establish proof of antibody specificity.

We caution that prior results reported in the literature using many of these c-FLIP antibodies may not be reliable. Incorrect recognition of c-FLIP may have adverse consequences on its potential use as a biomarker and target in cancer therapy.

In conclusion, the status quo of poor antibody characterization is a significant and ongoing problem resulting in mistaken interpretations and non-reproducible results that ultimately negatively impacts the rate of translation of preclinical drug targets into effective therapies. This is costly not only for the cancer patients who are awaiting better therapeutic strategies but also costly on our Federal, and non-Federal research funds. We strongly encourage higher standards for validation of all antibodies, that require genetic knockdowns and/or knockouts to establish proof of antibody specificity. .

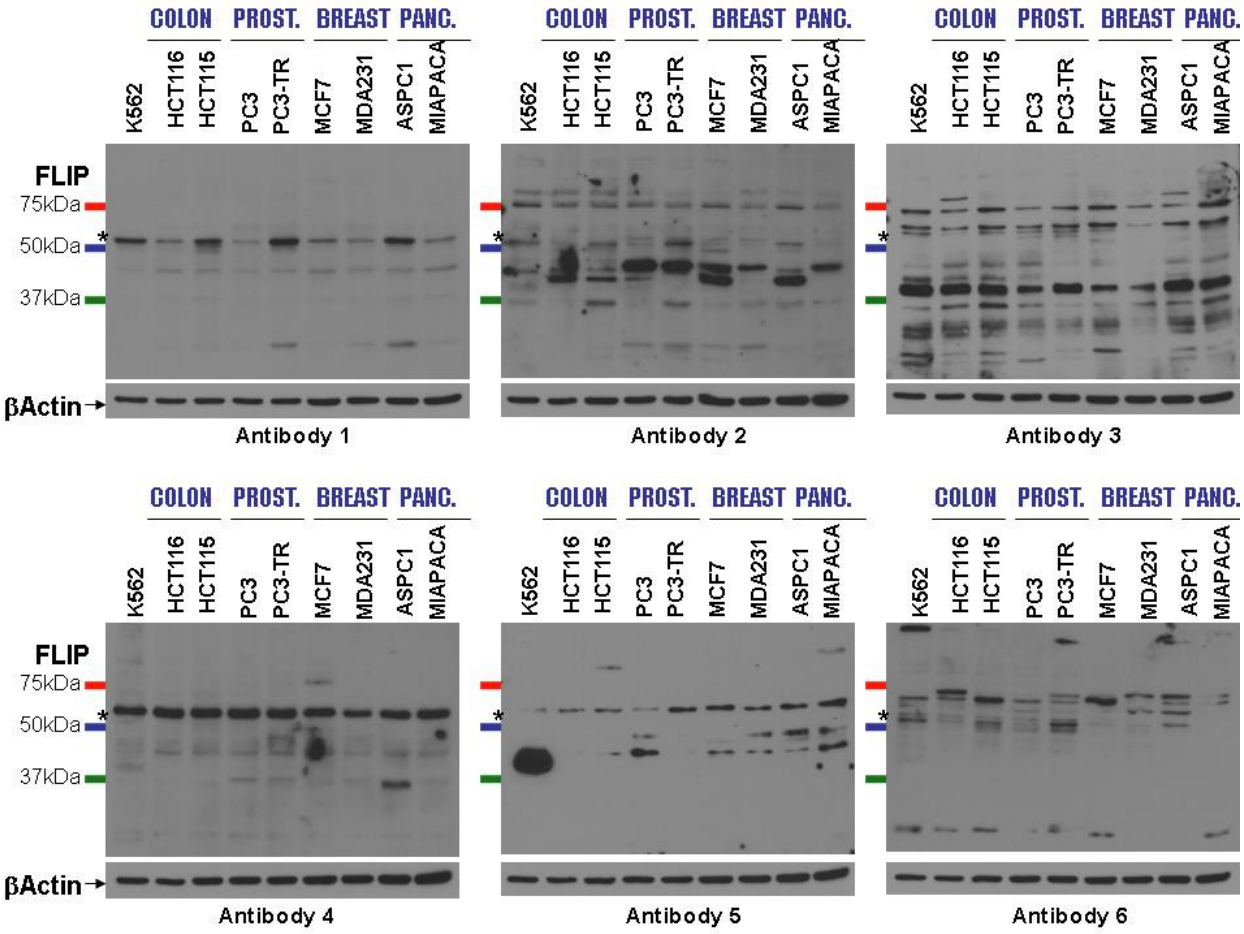



**Figure 12.** Detection of endogenous $FLIP_L$ expression levels with six different antibodies shows significant variation between the results obtained with each of the antibodies used. (**a**) Nine different cancer cell lines: K562 (leukemic cell line), HCT116, HCT115 (colon cancer cell lines), PC3, PC3-TR (prostate cancer cell lines), MCF7, MDA231 (breast cancer cell lines), ASPC1 and MIAPACA (pancreatic cancer cell lines) were analyzed by Western blot for endogenous $FLIP_L$ expression levels with six different antibodies;
βActin was used as a loading control; one of three independent experiments is shown; NT – N terminus; CT – C terminus; Prost. – Prostate; Panc. – Pancreatic; * - size of $FLIP_L$ (55kDa)

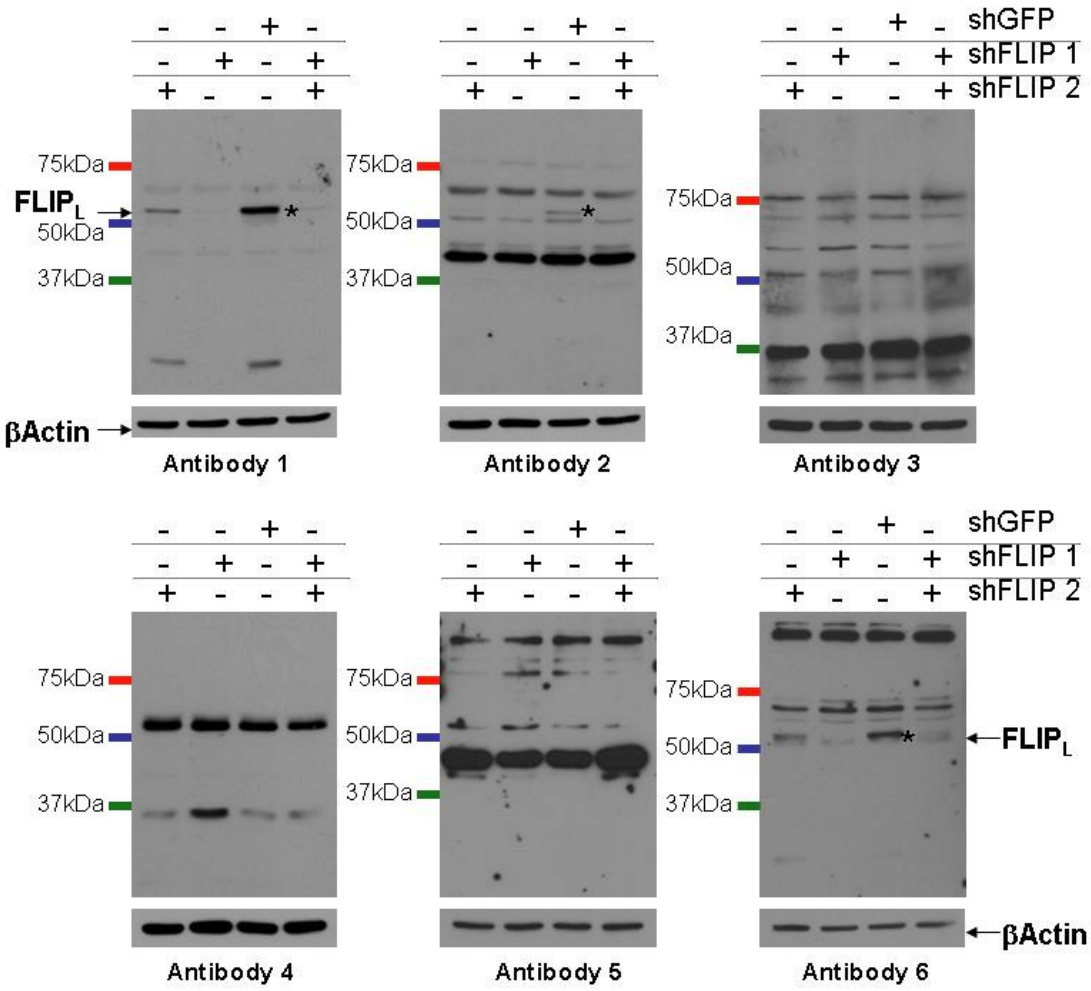



**Figure 13:** c-FLIP knockdown shows that only three of the six antibodies correctly identify endogenous c-FLIP in ASPC1 cells. ASPC1 pancreatic cancer cell line was analyzed by western blot for endogenous $FLIP_L$ expression levels with six different antibodies; In particular, two of these three antibodies (antibody 2 and 6) recognized high levels of non-specific proteins with molecular weights close to that of c-$FLIP_L$, rendering the identification of endogenous c-$FLIP_L$ nearly impossible in the absence of the knock-down/knock-out control experiments. βActin (beta Actin) was used as a loading control; one experiment from three different experiments is shown here; * - endogenous c-$FLIP_L$ detected by antibodies 1,2 and 6.

***In silico* identification of the small molecules that directly bind caspase 8 and potentiate TRAIL-induced cell death (selected figures are presented)**

Many cancer cells show resistance to TRAIL-induced apoptosis and efficient strategies for sensitization to TRAIL have to be developed[3]. The apoptosis process is triggered by recruitment of adaptor proteins, such as FAS-associated death domain (FADD), to the death receptors, which is followed by recruitment of caspase 8 pro-enzyme and formation of the DISC. This causes dimerization of caspase 8 resulting in its activation and in activation of caspase cascade. $cFLIP_L$ is homologous to caspase 8 but has a pseudocaspase domain which lacks catalytic residues[9]. Caspase 8-cFLIP heterodimers are formed at the DISC, when FADD recruits both FLIP and caspase 8 to its cytoplasmic tails. Active caspase 8 generated from an efficient DISC, can be inhibited by high levels of c-FLIP, antagonizing caspase 8 activation. Thus, one attractive strategy to sensitize resistant malignancies to TRAIL-induced cell death is the use of small molecules that target caspase 8 and promote caspase 8 activation in response to TRAIL, either by stabilizing the formation of caspase 8 homodimers or by inhibiting c-FLIP. For the first time, we describe the discovery and characterization of a small molecule that directly binds caspase 8 and enhances caspase 8 activation when combined with TRAIL, but not alone. The molecule was identified through an *in silico* chemical screen for compounds with affinity for the caspase 8 homodimer's interface. The compound was experimentally validated to directly bind caspase 8, and to promote caspase 8 activation and cell death in single living cells or population of cells, upon TRAIL stimulation. Our approach is a proof-of-concept strategy leading to the discovery of a novel small molecule that not only has activity in TRAIL-resistant cancer cells, but may also provide insights into the structure-function relationship of caspase 8 homodimers as putative targets in cancer.

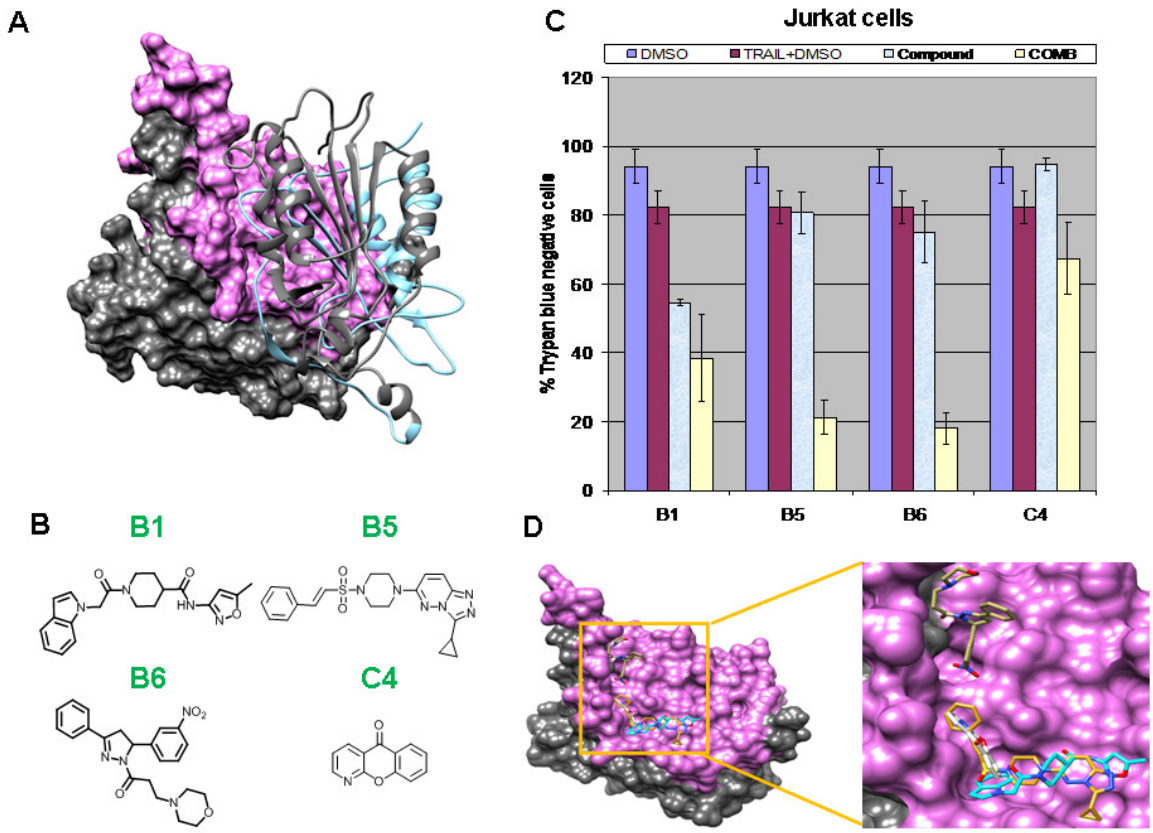



**Figure 14.** ***In silico*** **discovery of small molecules that bind caspase 8 and their experimental effect on Jurkat cells survival**

Zymogen caspase 8 interface region containing the small subunit (purple) was targeted and zymogen caspase 8 model (based on (PDB ID: 3H13) was used as the receptor for *in silico* molecular docking studies.

**A.** Zymogen caspase 8 homodimer showing the interface region with one caspase 8 monomer in surface representation and the other monomer in ribbon view. Different domains of the caspase 8 are distinguished by color: large subunits are shown in grey and small subunits shown in purple and light blue. A grid box was introduced spanning whole interface region for the search space.

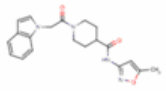
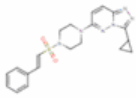
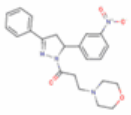
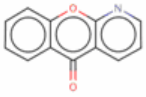
**B.** Compounds that experimentally sensitize Jurkat cells to TRAIL-induced apoptosis. Cell death/viability was evaluated in Jurkat cells by using an automated Trypan blue exclusion essay. Jurkat cells were treated with DMSO (control), TRAIL 7.5ng/ml, the compounds (80µM) and the combination, for 24h, in 1% FBS. Results show that all four compounds sensitize Jurkat cells to TRAIL-induced cell death, with compounds 2 and 3 being the most effective. First compound can also induce significant cell death when used alone.

**C.** The 2D structures of the hit compounds B1, B5, B6 and C4, which were experimentally validated to sensitize Jurkat cells to TRAIL-induced cell death.

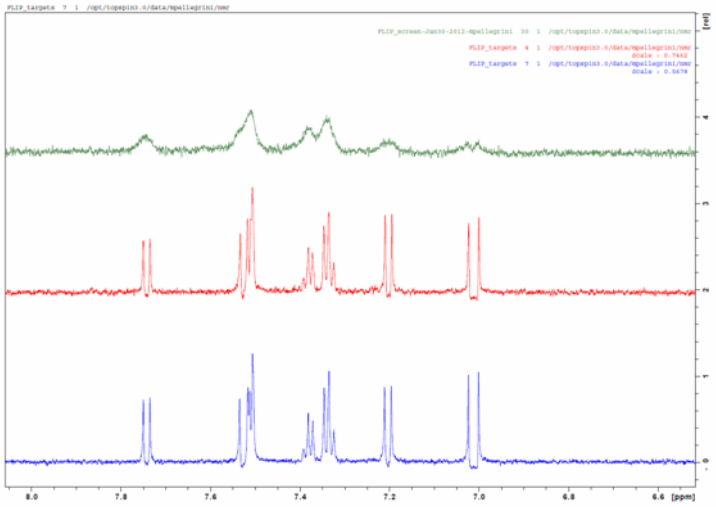
**D.** 3D conformation of the hit compounds bound to caspase 8 interface region. Note: compound 1 (cyan), compound 2 (gold), compound 3 (Khaki) and compound 4 (white). Right panel: enlarged boxed area of the left panel;

A.

## STD-NMR experiments results

| Target | B1 | B5 | B6 | C4 |
|---|---|---|---|---|
| |  |  |  |  |
| Caspase 8 | + v. small | broadening | - | - |
| FLIP | - | - | - | - |
| GST (Ctrl) | - | - | N/A | - |

B.



**GST-Casp8 + B5**

Green: B5 + GST-Casp8
Red: B5 in Casp-8 buffer
Blue: B5 + GST

**Figure 15. B5 small molecule compound directly binds caspase 8,** as determined by the STD-NMR experiments (see broadening of the spectrum for B5), while B1 may only bind caspase 8 with very low affinity, and B6 or C4 do not bind caspase 8.

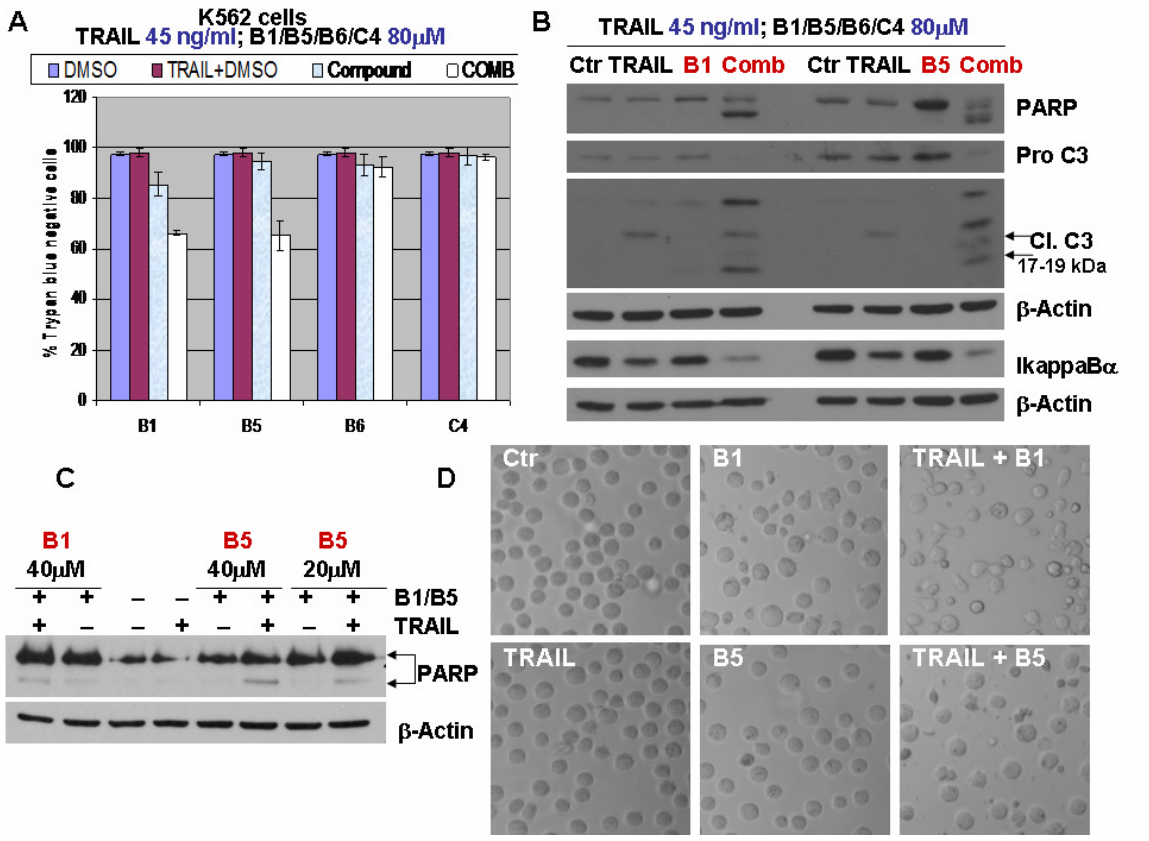



**Figure 16. The effect of B1, B5, B6 and C4 on sensitizing leukemic K562 cells to TRAIL-induced apoptosis; A.** K562 leukemic cells were treated with DMSO (control), 45 ng/ml TRAIL, 80 μM B1, B5, B6 or C4, and the combination, for 24h. Viability was measured by Trypan Blue dye exclusion method, using a TC10 Automated Cell Counter (Biorad, USA). The results represent the mean +/- standard deviations (SDs) of 3 independent experiments, each one with multiple replicates. The results demonstrate that only B1 and B5 compounds are effective in sensitizing K562 cells to TRAIL-induced cell death, while B6 and C4 do not show any effect at 80uM; **B.** K562 cells were treated with DMSO (control), 45 ng/ml TRAIL, 80 μM B1 or B5, and the combination, for 24h, followed by detection by western blot of the procaspase 3, cleaved caspase 3, PARP cleavage (which is caspase mediated) and IkappaBα levels. The combined regimen resulted in procaspase 3 and PARP cleavage, implying caspase 3 activation. The combinations also induce a significant decrease in the IkappaBα levels, protein which is known to keep NFkB in an inactive state, suggesting a potential activation of the NFkB pathway, a pro-survival pathway; **C.** K562 cells were treated with DMSO (control), 28 ng/ml TRAIL, 20u μM or 40 μM B5, 40 μM B1, and the combination, for 24h, followed by detection of the PARP cleavage. We clearly see that B5 at 20 μM and 40 μM, and B1 at 40 μM, sensitize K562 to TRAIL-induced PARP cleavage, which are indicative of caspase activation; **D.** K562 leukemic cells were treated with DMSO (control), 45 ng/ml TRAIL, 80 μM B1 or B5, and the combination, for 24h. Phase contrast microscope images of the untreated or treated cells are presented. A Nikon Eclipse TI-S Inverted Microscope was used (10X objective lens). While single treatments did not significantly change the morphology and number of the cells (except in the case of 80 μM B1, where some cells have apoptotic-like features), the combined treatment induced a change in morphology/shape and a decrease in the number of the cells; β-Actin was used as an internal loading control. A total of three independent experiments were performed for each panel.

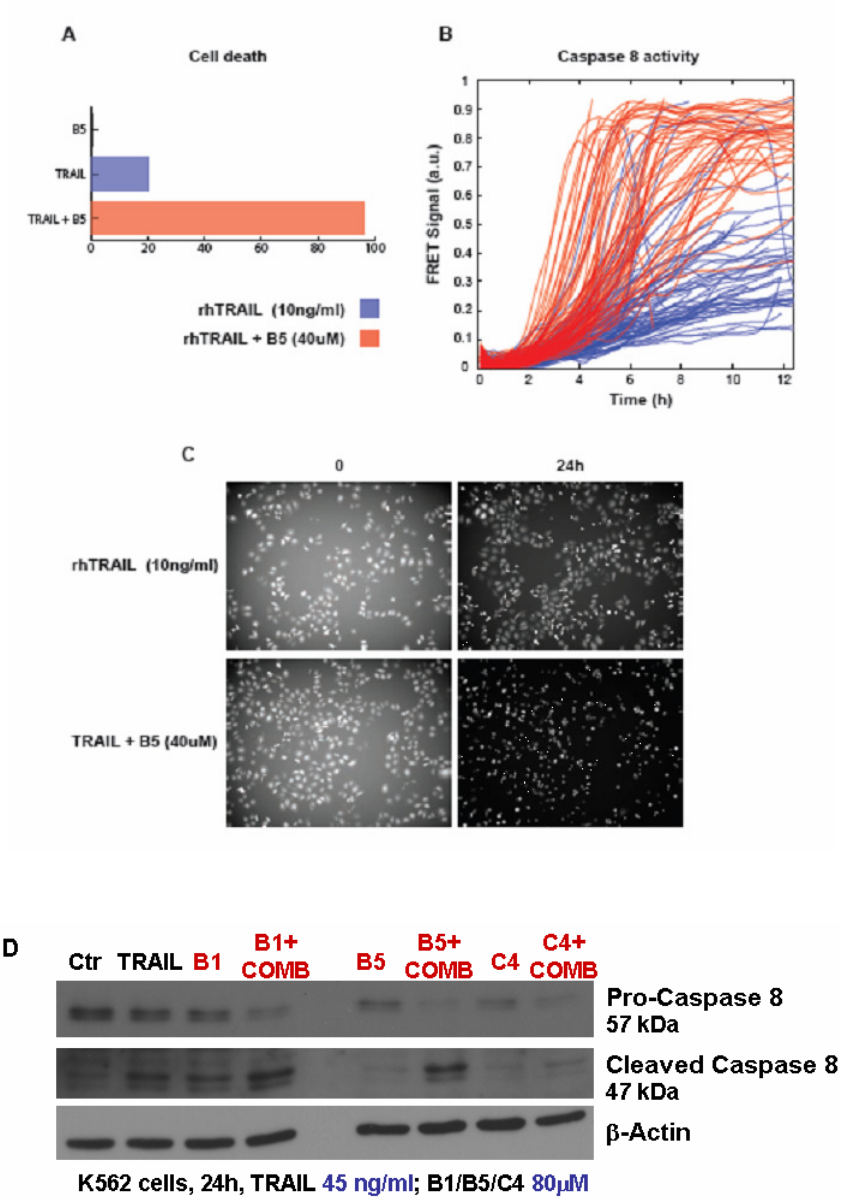



**Figure 17. B5 potentiates TRAIL-induced caspase 8 activation in *single cells* (A-C, Hela) and in population of cells (D, K562).**

**A-C** FRET detection of caspase 8 activation in a time-lapse experiment within *single living Hela cells*. Caspase 8 is activated faster and at higher levels by TRAIL when B5 (40μM) is present, in single cells.

**D**. K562 leukemic cells were treated with DMSO (control), 45 ng/ml TRAIL, 80 μM B1, B5 or C4, and the combination, for 24h, followed by detection by western blot of the procaspase 8 and cleaved caspase 8. While 80 μM B5 alone can't induce an increase in the cleaved caspase 8 (active caspase 8), B5 in combination with TRAIL increases TRAIL-induced caspase 8 activation. 80 μM B1 is able to induce some caspase 8 cleavage alone and can also increase the TRAIL-induced caspase 8 activation. 80 μM C4 does not have any effect on caspase 8 activation when used alone, and does not potentiate TRAIL-induced caspase 8 activation, in K562 cells.

# CONCLUSIONS

The work presented in this PhD thesis has led to several important conclusions, significant advances with great potential for the development of new anti-cancer therapeutics.

The experimental data brings new critical insights into the regulation and control of FOXO tumor suppressor, in particular of FOXO3. For the first time I identified PLK1 as a novel suppressor of FOXO3 and contributed to the discovery of PP2A as the first phosphatase for FOXO3. While PLK1, a critical kinase for cellular proliferation, can inhibit FOXO3 tumor suppressor and relocate it into the cytoplasm, where can be degraded, PP2A phosphatase dephosphorylates FOXO at the Akt-dependent phosphorylation sites, activating it and relocating it into the nucleus, where FOXO acts as a transcription factor for multiple pro-apoptotic (Bim, Trail) or cell cycle arrest (p27, p21) proteins. As FOXOs are inactivated in many malignancies by phosphorylation or by mutation (fusion mutants), finding novel ways of reactivating FOXOs represent promising strategies. Thus, modulation/targeting PLK1 that results in FOXO3 activation is a strategy that is worth investigating. Novel inhibitors of PLK1 are already available and their effect on cancer cells in vitro and in vivo is currently investigated. Moreover, PP2A activation results in multiple effects, one of them being FOXO3 activation.

Bcr-Abl activates many survival pathways, including PI3K-Akt, activation which results in inactivation of FOXO tumor suppressors (Akt phosphorylates and inactivates FOXOs). My interest in investigating novel strategies for targeting Bcr-Abl positive leukemias that are resistant to current Tyrosine Kinase Inhibitors resulted in the discovery of a combined regimen of bortezomib and mitotic inhibitors (known non-specific FOXO3 activators) that efficiently kills Tyrosin-kinase inhibitor sensitive and resistant Bcr-Abl-positive leukemic cells via down-modulation of BCR-ABL.

Last, but not least, acting downstream of FOXOs, we also found a novel small molecule that activates caspase 8. This small molecule (named by us B5) was designed to bind at the interface between caspase 8 and c-FLIP. c-FLIP protein is a major factor of resistance to apoptosis induced by many cytokine-/death ligand-based therapeutics and a potential theranostic biomarker. FOXO3 protein induces downregulation of c-FLIP protein

expression. This small molecule directly binds caspase 8 and overcomes the resistance to TRAIL-induced apoptosis in multiple cancer cells, including leukemic and prostate cancers cell lines, by potentiating TRAIL-induced activation of caspase 8.

Taken together, these discoveries not only represent important advances in understanding and characterizing FOXO tumor suppressor regulation and activity (PLK1 and PP2A identification as new partners of FOXOs) but also provide novel therapeutic strategies with great potential in Bcr-Abl positive leukemia (combined treatment of bortezomib with mitotic inhibitors) and in TRAIL-resistant malignancies (a novel small molecule that directly binds and activates caspase 8 sensitizing resistant cancer cell to TRAIL-induced apoptosis)

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# LIST OF PUBLICATIONS

**Overall number of citations (at the time of thesis submission)**:

349 citations- *Google Scholar*: http://scholar.google.com/citations?user=cLT3_JEAAAAJ&hl=en

217 citations - *ISI Web of Knowledge/ResearcherID*: http://www.researcherid.com/rid/G-2823-2010

**Selected articles** published or submitted/in preparation by the author representing the findings detailed in this PhD thesis:

**A. Published articles**

1. **Bucur O**, Stancu AL, Goganau I, Petrescu SM, Bertomeu T, Dewar R, Khosravi-Far R. Combination of bortezomib and mitotic inhibitors down-modulate BCR-ABL and efficiently eliminates Tyrosin-kinase inhibitor sensitive and resistant Bcr-Abl-positive leukemic cells. PLoS One 2013 Oct 14; 8(10):e77390; 3 citations (Google Scholar)

2. **Bucur O**, Pennarun B, Stancu AL, Nadler M, Muraru MS, Bertomeu T, Khosravi-Far R. Poor antibody validation is a challenge in biomedical research: A case study for detection of c-FLIP, Apoptosis 2013 Oct;18(10):1154-62; 1 citation (Google Scholar);

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5. Plati J, **Bucur O**, Khosravi-Far R. Apoptotic cell signaling in cancer progression and therapy. Integr Biol (Camb). 2011 Apr;3(4):279-96; 20 citations. The article was *named "Hot Review" and "highly recommended"* on the official website of Integrative Biology; 53 citations (Google Scholar);

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10. **Bucur O**, Ruspasinghe C, et al. Novel caspase 8 selective small molecule potentiates TRAIL-induced cell death (in preparation).

The following reviews/articles published by the author were used as the main source for:
**INTRODUCTION (Chapters I, II, III)**

- **Bucur O** et al, *Cell Death and Disease* 2012; (editorial)
- Plati J, **Bucur O** et al, *Integrative Biology* 2010; (review)
- Plati J*, **Bucur O*** et al, *J Cell Biochem* 2008; (review; * - equal contribution)
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- Pennarun B, Gaidos G, **Bucur O** et al, *Cell Death and Disease* 2013; (experimental article)

The following **experimental articles** were used as a main source for the
**RESULTS section (Chapters V, VI, VII, VIII)**

- **Bucur O** et al, *PloS One*, 2013 – Chapter V
- Singh A, Zhou S, **Bucur O** et al, *Mol Biol Cell*, 2012 – Chapter VI
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- **Bucur O** et al, *Apoptosis*, 2013 – Chapter VII
- **Bucur O** et al, in preparation – Chapter VIII