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Targeting LEDGF/p75 to Sensitize Chemoresistant Prostate Cancer Cells to Taxanes

Leslimar Rios-Colón

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Targeting LEDGF/p75 to Sensitize Chemoresistant Prostate Cancer Cells to Taxanes

by

Leslimar Rios-Colón

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Pharmacology

June 2017
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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Lubo Zhang, Professor of Pharmacology
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ABSTRACT OF THE DISSERTATION

Targeting LEDGF/p75 to Sensitize Chemoresistant Prostate Cancer Cells to Taxanes

by

Leslimar Ríos-Colón

Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, June 2017
Mentor: Dr. Carlos A. Casiano, Professor

Prostate cancer (PCa) is the second most diagnosed cancer in males. This disease disproportionately affects African American men, with a higher incidence and mortality compared to other ethnic/racial groups. An aging male population and the complexity of addressing the health disparities associated with this disease puts PCa into the spotlight due to its serious public health implications and the imminent fiscal challenge over the next decades. Chronic prostate inflammation resulting in activation of stress and pro-survival pathways contribute to disease progression and the development of chemoresistance. Lens epithelium-derived growth factor p75 (LEDGF/p75) is a stress-response protein that promotes cellular survival against environmental stressors, including oxidative stress, radiation, and cytotoxic drugs. It is overexpressed in PCa and other cancers and has been associated with features of tumor aggressiveness, including resistance to cell death and chemotherapy. This research work shows that the endogenous levels of LEDGF/p75 are upregulated in metastatic castration resistant prostate cancer (mCRPC) cells selected for resistance to the taxane drug docetaxel (DTX). These cells also showed resistance to the taxanes cabazitaxel (CBZ) and paclitaxel (PTX), but not to the classical inducer of apoptosis TRAIL. Silencing LEDGF/p75 effectively sensitized
taxane-resistant PC3 and DU145 cells to DTX and CBZ, as evidenced by a significant decrease in their clonogenic potential. While TRAIL induced apoptotic blebbing, caspase-3 processing, and apoptotic LEDGF/p75 cleavage, which leads to its inactivation, in both taxane- resistant and -sensitive PC3 and DU145 cells, treatment with DTX and CBZ failed to robustly induce these signature apoptotic events. Also, pre-treatment with caspase inhibitor zVAD partially rescued the cells from TRAIL-induced cell death. These observations suggested that taxanes induce both caspase-dependent and -independent cell death in mCRPC cells, and that maintaining the structural integrity of LEDGF/p75 is critical for its role in promoting drug-resistance. We also report the initial screening and selection of candidate small molecule inhibitors (SMIs) to target this protein and sensitize taxane-resistant cells to chemotherapy.
CHAPTER ONE
INTRODUCTION

Prostate Cancer and Available Therapies

Prostate cancer (PCa) accounts for 1 in 5 new cancer diagnoses in men in the United States, and is the most frequently diagnosed cancer in this population, with 161,360 new cases and 26,730 deaths projected for 2017 [1]. Furthermore, it has been extensively documented that this malignancy disproportionately and aggressively affects African American men [2-3]. This group is more likely to be diagnosed with PCa, with an estimated 1 in 6 African American males being diagnosed over their lifetime compared to 1 in 8 Non-Hispanic white males. Also, they are more likely to be diagnosed at an advanced stage and present a lower-stage five-year relative survival, with a 2.4 higher mortality rate compared to Non-Hispanic whites [2, 4]. Although they are the group most affected by this disease, African Americans are still underrepresented in potentially life-saving clinical trials [5]. An aging male population and the complexity of addressing PCa mortality disparities puts this malignancy in the spotlight due to the serious public health and fiscal challenge it will pose over the next decades [2-6].

Although PCa is treatable in its earlier stages, resistance to chemotherapeutic drugs is a major deterrent to effectively treat the advanced form of the disease, known as castration resistant prostate cancer (CRPC). Previously known as hormone-resistant prostate cancer, CRPC is characterized by disease progression despite of castrate levels of circulating testosterone [7]. Treatment options for CRPC include systemic chemotherapy with the taxanes docetaxel (DTX) and cabazitaxel (CBZ) [7-8].

Taxanes prevent the disassembly of the microtubules [9]. These are essential
components of the cytoskeleton involved in many vital cellular functions such as chromosome separation, cell shape, vesicle transport, transcription factor trafficking, mitochondrial functioning, adaptation to the microenvironment, and cell signaling [9, 10]. Treatment with taxanes results in cell cycle arrest and cell death [9]. Paclitaxel (PTX) was the first taxane to become clinically available as a single-agent and is currently utilized for the treatment of ovarian, breast and non-small-cell lung cancers, as well as Kaposi’s sarcoma [11, 12]. It’s not widely utilized for the treatment of CRPC since the results of phase II clinical trials were modest and there were not phase III trials to test for the efficacy of this drug alone or in combination with another microtubule inhibiting agent, entramustine [11]. This taxane features a common tetracyclic baccatin core with a side chain at C13, a benzyl amide moiety at C3, a hydroxyl at C7 and an acetyloxy at C10 [12]. The chemical structure of PTX is shown in Figure 1.

Docetaxel (DTX) is a semisynthetic taxane that contains a benzyl amide moeity in the position C3 substituting the bulky benzyl amide moiety, and that showed approximately twice the efficacy of PTX in pre-clinical models (Figure 1) [11, 12]. The use of DTX (75 mg/m² every 3 weeks) plus prednisone (10 mg/day) is the first-line therapy currently recommended by international guidelines for patients with symptomatic CRPC [13]. In a randomized trial, TAX 327, treatment with DTX showed an average overall survival (OS) of 18.9 months compared to the group treated with the drug mitoxantrone which showed an OS of 16.5 months [13, 14]. Although DTX is extensively utilized as the first-line chemotherapy treatment for CRPC, there are still concerns about the variable response in patients, initial resistance to the drug, and serious adverse effect [15].
Cabazitaxel (CBZ) is a dimethyloxy derivative of docetaxel (Figure 1) with less affinity to the multi-drug resistance protein P-glycoprotein and with an increased penetration potential of the blood-brain barrier in preclinical models [16]. Like PTX, both DTX and CTX have a common tetracyclic baccatin core structure with a side chain at C13, but differ at C7 and C10 since the hydroxyl groups in DTX are substituted by acetoxy groups in CBZ (Figure 1) [12]. In the TROPIC clinical trial, CBZ (25 mg/m²) plus prednisone (10 mg/day), showed benefits in patients compared to the group treated with mitroxantone with OS of 15.1 and 12.7 months, respectively [17]. Before the approval of CBZ by the FDA in 2010, second-line treatment options after development of resistance to DTX were limited to palliative care with little OS benefit [16].

It is also important to consider the role of androgen receptor (AR) activation in the development of advanced disease even after progression to CRPC. Mechanisms including increased AR synthesis, mutation and splice variants, overexpression and its co-activators, and crosstalk between AR signaling and other signal transduction pathways such as glucocorticoid signaling can increase AR activation with marginal levels of androgens, thus representing a promising treatment strategy for advanced disease [17]. Androgen deprivation therapies (ADT) with enzalutamide or abiraterone acetate plus prednisone, and other non-ADT therapies such as sipuleucel-T and radium-223, are now available to patients with CRPC, with average overall increase in survival of 3-5 months and improvement in relevant secondary end-points (Figure 2) [14]. Unfortunately, CRPC is still poorly managed and most patients succumb to the disease within 3 years of diagnosis [14, 15]. Development of primary resistance and cross-resistance are not uncommon, [7, 15] and exposure to ADT itself may lead to metastatic disease [15].
Figure 1. Chemical structure of PTX, DTX and CBZ. These taxanes have a common tetracyclic baccatin core with a side chain at C13, but have functional group modifications three sites. (Adapted from Churchill et al, 2015) [12].
Figure 2. Currently available treatment options for CRPC (adapted from Fujimoto et al, 2016) [17].
Inflammation and Prostate Cancer

A recent study linked the role of chronic inflammation in benign prostate tissue with increased odds of developing high grade PCa compared to those patients with no histological evidence of inflammation [18]. Growing evidence suggests that inflammation plays a role in the regulation of the tumor microenvironment and the disruption of the anti-tumor immune response, leading to the development of PCa [19, 20]. Also, DNA damage may result from inflammation-induced oxidative stress leading to gene rearrangements and other anomalies associated with aggressive disease [21]. Thus, the identification of proteins involved in resistance to inflammation-induced oxidative stress is not only critical to understand the molecular pathogenesis of PCa, but also to develop individualized treatment strategies to improve prognosis in CRPC patients [22].

The Lens Epithelium-Derived Growth Factor of 75kD

Lens Epithelium Derived Growth Factor of 75 kD (LEDGF/p75) has recently emerged as a stress protein that promotes cellular survival against many different environmental stressors, including oxidative stress, radiation, heat, serum starvation, and cytotoxic drugs such as DTX, particularly in cancer cells [23-35]. Also known as PC4 and SFRS1 interacting protein (PSIP1), and dense fine speckled autoantigen of 70 kD (DFS70), this protein has recently attracted considerable attention due to its broad relevance to cancer, autoimmunity, eye diseases, and HIV-AIDS [29, 30]. LEDGF/p75 is the target of autoantibody responses in a subset of patients with PCa, as well as in patients with diverse chronic inflammatory conditions and some apparently healthy individuals [29, 36-37]. While early studies suggested that LEDGF/p75 was a growth factor critical for the proliferation of lens epithelial cells [23], subsequent studies have
demonstrated that this protein is not a lens specific growth factor but rather a ubiquitous nuclear transcription co-activator with oncogenic functions that is activated during the cellular response to stress [29, 30]. Over the past decade LEDGF/p75 has also emerged as a critical cellular factor for the integration of the human immunodeficiency virus 1 (HIV-1) [38-43]. Through interaction with the HIV integrase (HIV-IN), LEDGF/p75 facilitates the integration of HIV-1 into host chromatin [44].

**LEDGF Structure and Function**

The *PSIP1* gene has been mapped to chromosome 9p22.2 and encodes various splice variants of LEDGF [23], but the two more commonly recognized are LEDGF/p75 and its short alternative spliced variant LEDGF/p52 (Figure 3) [29]. Both LEDGF splice variants belong to the hepatoma derived growth factor (HDGF) family [26, 45], with a characteristic highly conserved PWWP domain, defined by the presence of the proline-tryptophan-tryptophan-proline amino acid sequence motif. The *PSIP1/LEDGF* gene is constituted by 15 exons and 14 introns, with exons 1-15 (530 amino acids) encoding LEDGF/p75 and exons 1–9 and 24 nucleotides of intron 9 encoding LEDGF/p52 (333 amino acids). The intron-derived carboxyl (C)-terminal tail-terminal tail (CTT, amino acid residues 326-333) is uniquely present in LEDGF/p52 but not in LEDGFp75. Although the function of this unique CTT is not completely clear, previous studies from our group implicated it in the pro-apoptotic function of LEDGF/p52, which, unlike LEDGF/p75, does not appear to have cellular survival functions [63]. Both splice variants of the *PSIP1/LEDGF* gene share the amino (N)-terminal residues 1-325, where the PWWP domain is located (residues 1-98) [23, 26, 45-46].
Figure 3. Representation of the two main splice variants of LEDGF, p75 and p52 (adapted from Ochs et al, 2016) [29].
The PWWP domain is a highly conserved region in members of the hepatoma
derived growth factor (HDGF) family and is functionally relevant to both splice variants
since evidence suggest its role in DNA binding, transcriptional repression, and
methylation [26, 45-47]. This domain facilitates the chromatin recognition, dynamic
interaction, and locking of LEDGF/p75 when this protein has interacting proteins bound
to its C-terminus [48]. In addition, the PWWP domain of LEDGF/p75 specifically
recognizes trimethylated histone H3K36, suggesting binding of this protein to
transcriptionally active regions in the chromatin [49]. Solution structure analysis of the
LEDGF/p75 PWWP domain revealed two distinct functional interfaces: a hydrophobic
cavity that interacts with the histone H3 tail containing trimethylated Lys36 (H3K36me3)
as well as a basic surface that binds non-specifically to DNA [50]. This cooperative
binding may be critical for high-affinity binding to chromatin since nucleosomal DNA
enhanced the binding affinity by 10,000 fold [51].

Other important structural features that are shared by the N-termini of
LEDGF/p75 and LEDGF/p52 are three charged domains (CR1, CR2, CR3), a nuclear
localization signal (NLS) (residues 148-156), a TAT- like sequence within the NLS, and
a basic leucine zipper (zip) sequence that overlaps with a helix-turn-helix (HTH) region
(residues 154-175) [28, 40, 52] (Figure 3). This zip sequence also overlaps with two AT-
hooks (residues 178-183 and 191-197). Functionally, AT-hooks bind to the minor
groove of DNA rich in AT nucleotide sequences and evidence suggests that these might
facilitate accessibility of promoters to transcription factor by modifying the architecture
of DNA [49]. The tripartite region, consisting of the NLS and AT-hooks are sufficient
for chromatin binding [52]. The second charged region (CR2), also designated the
supercoiled DNA recognition domain (SRD; residues 200-336), allows LEDGF/p75 to preferentially bind to active transcription sites in the negatively supercoiled DNA over unconstrained DNA [53]. The region comprised of residues 137–206 has been termed as non-specific DNA-recognition domain (NRD) [53].

The C-terminal region of LEDGF/p75 (residues 339-442), which is absent in LEDGF/p52, shares amino acid sequence homology with the HDGF-related protein 2 (HRP-2) and has been identified as the HIV-1 IN binding domain (IBD) [40, 54]. The IBD overlaps almost entirely with the epitope recognized by human autoantibodies recognizing LEDGF/p75, which are produced by healthy individuals as well as patients with different inflammatory conditions and some patients with PCa [29, 55]. Both the N- and C-terminal regions of LEDGFp75 are necessary for its transcription and stress survival functions by engaging in interactions with chromatin- binding proteins, or by binding to promoters regions of specific stress genes [42, 56-61]. Secondary amino acid sequence structures in the C-terminus of LEDGF/p75 are in their majority large random coiled regions containing disordered regions, implicated in the recognition of DNA and RNA, modulation of protein binding, and regulation of protein lifetime [62]. In addition, PSIPRED protein structure prediction server V2.1 and PHDsec programs predicted a LEDGF/p75 secondary structure consisting of N-terminal β-strand and α-helix [26].

The interaction of the LEDGF splice variants with multiple proteins or DNA to form an interactome suggests its significance in multiple processes that include proliferation, growth, differentiation, and cell survival and death [29]. There are studies reporting differences in the roles and interactions of LEDGF splice variants depending on the cellular context. For example, the two isoforms interact differently with proteins
involved in mRNA processing such as the Serine/arginine-rich splicing factor 1 (SRSF1) [49]. Our group also reported the antagonistic roles of LEDGF/p75 and LEDGF/p52 in the context of cell death [63]. During caspase-dependent cell death, p52 is cleaved by caspase-3 into fragments p48 and p38. Particularly, p38 lacks the PWWP domain at the N-terminus and is unable to transactivate the promoter (pr) region of heat shock protein 27 (HSP27), a well-known target gene of LEDGF/p75 [63] (Figure 4A). This cleavage fragment is however able to repress the HSP27pr transactivation potential of LEDGF/p75 and retains some of its chromatin association abilities. Interestingly, apoptosis can be induced when either p52 or its variants lacking most of the PWWP domain are overexpressed in tumor cell lines [63]. Our group also showed that cleavage of LEDGF/p75 during apoptosis by caspases-3 and -7 into a prominent fragment of 65 kD not only abrogated its pro-survival functions but also accelerated cell death under starvation conditions (Figure 4B) [26].

There is evidence suggesting that there are other genetic variants of LEDGF/p75 different from LEDGF/p52. Some of these have been identified in leukemic cells and thought to play different roles in apoptosis and cell survival [32]. Other variants derived from gene polymorphisms may affect the outcomes and pathogenesis of HIV-1 [64]. For instance, a study revealed that two LEDGF/p75 exonic variants, I436S and T473I were identified from a cohort of HIV-1 long-term nonprogresor patients [65]. However, further characterization revealed that these variants did not alter the interaction of LEDGF/p75 with HIV-1 IN [65].
Figure 4. Caspase mediated cleavage of LEDGF/p52 (A) and LEDGF/p75 (B) during apoptosis. (Adapted from Brown et al, 2008 and Wu et al, 2002) [26, 63].
Cellular Protective and Survival Functions of LEDGF/p75

Multiple studies have demonstrated the cellular protective functions of LEDGF/p75 upregulation against cellular stress inducers that may lead to an augmented state of oxidative stress, such as ultraviolet B (UVB) irradiation, hydrogen peroxide, alcohol, hyperthermia, nutrient deprivation, and some chemotherapeutic drugs [25-28, 32-35, 66-69]. LEDGF/p75 is a transcription factor adaptor with chaperone and chromosome-docking properties [48, 70], and evidence suggests that this protein aids in the transcription and activation of stress, antioxidant, and other protective and stress-survival genes [27, 71-79].

LEDGF/p75 is emerging as an important player in carcinogenesis as cells upregulate this stress-induced protein to evade cell death, induce favorable growth signals, and upregulate oncogenic proteins to maintain pro-survival functions such as cellular repair, protection from DNA damage, lysosomal stability, proteolytic evasion, and angiogenesis [33-34, 71, 77, 80]. Upon cellular exposure to a damaging agent that induces oxidative or thermal stress, LEDGF/p75 is upregulated and binds to heat shock elements (HSE) and stress response elements (STRE) in the promoter regions of specific target genes [27]. This transcription co-activator enhances the transactivation of antioxidant genes like albumin (ALB), thyroid peroxidase (TPO), superoxide dismutase 3 (SOD3), cytoglobin (CYGB), and antioxidant protein 2/peroxiredoxin 6 (AOP2/PRDX6), thus reducing the oxidative damage caused by oxidative stress and helping cells (both normal and cancer) evade cell death [79, 81]. In addition, LEDGF/p75 enhances the transactivation of anti-apoptotic proteins HSP27 and αB-crystallin that respond to stress-related damage by inhibiting caspase-3 activation [27, 82-83]. LEDGF/p75 also
transactivates the vascular endothelial growth factor C (VEGF-C), a prosurvival protein involved in angiogenesis and cancer metastasis [77, 84].

Several studies have also linked LEDGF/p75 aberrant expression to human leukemogenesis [85]. LEDGF/p75 expression was upregulated in chemoresistant blasts obtained from acute myeloid leukemia (AML) patients [32]. In addition, a chromosomal translocation associated to leukemogenesis, t(9;11)(p22;p15), results in a fusion of the N-terminal part of the nucleoporin 98 protein (NUP98) to the C-terminus of LEDGF/p75 [86-91]. LEDGF/p75 has also been identified as a key co-factor of the mixed lineage leukemia (MLL)–fusion complexes [85]. Specifically, the interaction of LEDGF/p75-NUP98 with menin-MLL complexes in leukemic cells targets the homeobox HOX genes that are normally associated with stem cell self-renewal, a function exploited by migrating cancer progenitors [59, 92]. The role of LEDGF/p75 in leukemia is discussed in more detail below.

**Target Genes of LEDGF/p75**

Detailed studies of LEDGF/p75 target genes are necessary to further understand its protective and cellular functions. Although it was initially reported that LEDGF/p75 interact with the HSE and STRE in promoter regions of its target genes [27, 61], further studies from our group and others failed to confirm these findings [52, 82]. Recently, studies using the DamID technology focusing on the highly annotated ENCODE (encyclopedia of DNA elements) region, demonstrated that LEDGF/p75 binds to DNA primarily downstream of the active transcription unit start sites. This binding is not restricted to STREs, and correlates with active chromatin markers and RNA polymerase
II binding [93]. We have to consider the possibility that LEDGF/p75 interacts with a particular motif in a promoter region depending on the cellular microenvironment and/or stress conditions. For instance, LEDGF/p75 might interact with a particular promoter element (such as STRE) of a certain target gene, for example HSP27, in a particular cellular system under conditions of stress such as drug treatment [82]. This would explain the differences observed in these previous studies, since not all were conducted under conditions of stress.

It remains to be established if the binding of LEDGF/p75 to specific promoter elements in its target genes occurs in different cellular contexts (e.g. cancer vs non-cancer, stress vs non-stress, microenvironment). As mentioned above, various studies have identified several target genes of LEDGF/p75, including HSP27 [27, 82], αB-crystallin [27, 76], gamma-glutamylcysteine synthetase [68], AOP2/PRDX6 [71], involucrin [72], alcohol and aldehyde dehydrogenases [75], VEGF-C [77-78], and interleukin 6 (IL-6) [94-95]. It is not clear, however, what are the specific microenvironmental, cellular, and molecular contexts that determine how and when LEDGF/p75 transactivates each one of these genes.

To identify additional target genes regulated by LEDGF/p75, our group utilized pathway specific gene expression profiling analysis in PC3 prostate cancer cells transiently depleted of this protein or stably overexpressing it [79]. Our study identified five oxidative-stress genes that exhibited statistically significant mRNA down- and up-regulation (fold-change ≥ 2) in response to LEDGF/p75 knockdown and overexpression, respectively. These were: CYGB, phosphoinositol-binding protein (PIP3-E/IPCEF-1), SOD3, TPO, and ALB. From this group, CYGB was selected for further validation and
characterization by immunoblotting due to its significance as a tumor suppressor gene, depending on context, and emergence as a cancer-associated stress protein [96-98]. Our results showed that LEDGF/p75 mRNA and protein expression in PCa cells paralleled those of CYGB [79]. Interestingly, our findings have been corroborated by a report that validated the elevated levels of CYGB protein in prostatic atrophy as compared to benign non-atrophic epithelium, prostatic intraepithelial neoplasia (PIN) or cancer [99]. This report proposed that CYGB has a protective function in preventing DNA damage induced by reactive oxygen species (ROS), aiding in the progression from inflammatory atrophy lesions to invasive carcinoma.

Other studies revealed that in leukemic cells LEDGF/p75 also regulates the expression of some HOX gene family members [59, 100]. It is plausible that gene regulation by LEDGF/p75 is dependent on cell type, stressor type and the microenvironment, as previously observed.

**Interacting Partners of LEDGF/p75**

LEDGF/p75 interacts with specific transcription factors to facilitate the transactivation of stress and cancer-related genes through both its PWWP domain and the C-terminal IBD region. Through its IBD region, LEDGF/p75 binds to and tethers MYC-binding protein JPO2 to chromatin, hinting a plausible role in the MYC-regulatory network, important in human cancers [42, 46, 57, 60]. As a result of ROS accumulation in the cell, LEDGF/p75 interacts with transcription factors such as JPO2 and PC4 transcription factor, pogo transposable element-derived protein with zinc finger (PogZ) [56], and cdc7 activator of S-phase kinase (ASK) [58]. These complexes then interact
with the promoter sites of antioxidant, anti-apoptotic, and other prosurvival genes. In leukemia cells, LEDGF/p75 interacts with the menin/MLL-HMT (mixed linkage leukemia-histone methyltransferase) transcription complex to transactivate cancer-associated genes and facilitate leukemic transformation [59].

The PWWP domain has been implicated by our group in the binding of MeCP2 (methyl CpG binding protein 2) to both LEDGF/p75 and LEDGF/p52 [82]. This interaction occurs via the PWWP-CR1 domain found in the N-terminal region of LEDGF/p75 and differentially regulates the HSP27 promoter in cancer cells [82]. Other proteins that interact with LEDGF/p75 through the PWWP domain are the transcription activator TOX4 and the splicing co-factor NOVA [101]. In addition, LEDGFp75 also assists in the recruitment of polycomb group protein Bmi1 and co-repressor Ctbp1 to MLL complexes in the HOX gene promoters [102].

**Regulation of LEDGF/p75 Expression and Function**

Studies were undertaken to find regulatory regions within the LEDGF/p75 promoter, using promoter truncations in reporter assays. These concluded that LEDGF/p75 TATA-Less promoter activation is driven by the transcription factor Sp1 since its increased expression leads to LEDGF/p75 upregulation, while its inhibition represses this upregulation [61, 103]. Regulation of LEDGF/p75 may involve posttranslational modifications by the small ubiquitin-like modifier 1 (SUMO1), with SUMOylation and deSUMOylation acting as a molecular switch that regulates the DNA-binding and transactivation capacity of LEDGF/p75 [104-105]. In a study using lens epithelium cells exposed to UVB, Sp1 binding was disrupted by a histone
deacetylase/histone methylase (HDAC1/ SUV39H1) complex recruited to the Sp1 regulatory region in the LEDGF/p75 promoter. This resulted in repression of LEDGF/p75 expression, leading to cell stress and death [106].

The transcriptional activities of LEDGF/p75 are also abrogated by the binding of transforming growth factor-β1 (TGF-β1) to its promoter region in lens cells [74]. This observation was consistent with studies using Prdx6−/− knockout mouse cells in which increased levels of TGF-β1 resulted in decreased LEDGFp75 expression [88]. This inverse relationship was also observed by Kubo and colleagues in diabetic and galactosemic cataractous rat lenses [107].

The pro-survival protein B-cell leukemia/lymphoma protein 2 (Bcl-2) was reported to significantly downregulate the expression of endogenous αB-crystallin gene by decreasing the transcriptional and DNA binding activity of LEDGF/p75 [108]. In a separate study, the same group showed that Bcl-2 activates the ERK signaling pathway to downregulate LEDGF/p75 transcriptional activity, resulting in reduced expression of αB-crystallin [109]. Kubo and colleagues corroborated this negative relationship between LEDGF/p75 and the TGF-β and Bcl-2 proteins in their gene microarray analysis [107].

Evidence from our group also suggests that caspase-dependent apoptotic cell death appears to play a role in the regulation of LEDGF/p75 pro-survival functions. As shown above in Figure 4, during apoptosis caspase-3 cleaves LEDGF/p75 at specific aspartic acid residues located in the N-terminal PWWP domain and the C-terminal region into prominent fragments of 65 and 58 kD [26, 35, 63]. This apoptotic cleavage impairs the protein’s stress survival activity, resulting in increased cell death. The pro-survival functions of LEDGF/p75 are also negatively regulated by the alternative splicing of the
PSIP1/LEDGF gene, since p52 antagonizes its transcriptional activity and induces apoptosis in cancer cells [63]. As mentioned previously, our group observed that during apoptosis p52 is also cleaved by caspase-3 to generate a p38 fragment that antagonizes the transcriptional function of LEDGF/p75 [63]. Ectopic overexpression of LEDGF/p52 resulted in decreased cell viability as a result of caspase-dependent cell death that resulted in LEDGF/p75 cleavage [63].

LEDGF/p75 transcription is also regulated by micro-RNAs (miRNAs). In one study miR-155 was induced in macrophages stimulated with lipopolysaccharide, resulting in down-regulation of LEDGF/p75, and ectopic expression of this miRNA reduced LEDGF/p75 transcript [110]. In a separate study it was demonstrated that miR-135b also down-regulated LEDGF/p75 both in human cell lines and in murine vestibular sensory epithelia of the inner ear [111].

The PWWP domain was reported to also interact with partners possibly regulating its interaction with chromatin [101]. Thirteen potential partners were originally identified using yeast-two-hybrid, but TOX4 and NOVA1 were selected for further studies. Both proteins have DNA and RNA binding properties and are involved in transcriptional regulation [101]. TOX4 and NOVA1 co-localized with LEDGF/p75 in different cell lines. Three PWWP interacting regions (PIRs) of TOX4 and NOVA1 proteins were found to interact with the PWWP domain of LEDGF/p75. They also observed a reduction vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV infection in cells transiently expressing Flag-tagged PIRs of TOX4 and NOVA1. This effect was possibly due to the inhibition of the LEDGF/p75-IN interaction [101].
Finally, the crosstalk between LEDGF/p75 and proteins involved in inflammation has been explored, suggesting a possible role of this protein in regulating inflammatory pathways, or, alternatively, these pathways regulating LEDGF/p75. For instance, Takeichi and colleagues investigated the relationship between IL-6 and LEDGF/p75 in HaCaT cells [94, 95]. Their studies revealed that overexpression of LEDGF/p75 induced the MK2/IL-6/STAT3 (signal transducer and activator of transcription 3) signaling pathway via p38 phosphorylation, whereas its depletion reduced IL-6 levels.

Another study involving a microarray analysis after induction of STAT3β in breast cancer cells, which acting as a dominant-negative regulator promoted apoptosis, resulted in repressed LEDGF/p75 expression [112]. Interestingly, overexpression of this survival protein rescued the cells from STAT3β’s effect on cell viability, suggesting a negative feedback mechanism [112]. Numerous studies have suggested involvement of IL-6 and its receptor (IL-6R) in cellular/molecular events in which LEDGF/p75 is also involved such as drug resistance, anti-apoptotic effects, tumor progression, and development of CRPC under androgen deprivation [113-115]. These events also appear to be regulated through (STAT3) and Bcl-xl related pathways [116].

Future studies using RNA interference, chromatin immunoprecipitation (ChIP) analysis, transcription reporter assays, and electrophoretic mobility shift assays (EMSA) should be conducted to establish unambiguously whether IL-6 and other inflammatory cytokines are target genes of LEDGF/p75.

**Autoantibody Responses to LEDGF/p75 in Cancer and Inflammatory Conditions**

The presence of autoantibodies to LEDGF/p75 (referred to as the DFS70
autoantigen in the field of autoimmunity) in apparently healthy individuals and in a variety of inflammatory conditions has been thoroughly documented by our group and others [29, 30, 37]. The immune system responds to changes in the cellular microenvironment such as the up-regulation of tumor-associated antigens (TAAs), production of splice variants or cleaved fragments, and changes in the cellular concentration, distribution, localization, or excretion of specific endogenous proteins by producing antibodies to these autoantigens in genetically susceptible individuals [132].

Up-regulation of LEDGF/p75 in response to chronic inflammation or malignant transformation is thought to lead to increased concentration, distribution, an aberrant immune presentation of this protein, consequently resulting in T cells recognizing this protein as abnormal and triggering B cells to produce an autoantibody response [132-133]. This increased immunogenicity and autoantibody recognition of LEDGF/p75 has been reported in patients with certain inflammatory conditions such as atopic dermatitis, with cataracts and alopecia areata, as well as in PCa [36, 134-136]. Interestingly, LEDGF/p75 autoantibodies from patients with atopic dermatitis and cataracts were reported to be cytotoxic to the crystalline lens and lens epithelial cells [134].

The clinical significance of LEDGF/p75 autoantibodies still remains unclear, since these antibodies have been identified in several autoimmune diseases at lower frequencies than sera from individuals with no evidence of autoimmune disease [137-139]. This lower frequency of LEDGF/p75 autoantibodies in patients with systemic autoimmune rheumatic diseases (SARD) compared to apparently healthy individuals has recently led to the notion that anti-LEDGF/p75 autoantibodies could be utilized as biomarkers to rule out a SARD diagnosis [140].
In an early study, 597 self-reported healthy hospital workers were screened for the presence of antinuclear autoantibodies (ANAs) and observed that 54% of all ANA-positive apparently healthy individuals had anti-LEDGF/p75 antibodies [139]. The conclusion was that this could be a naturally occurring autoantibody in both healthy and diseased individuals, not associated with a specific pathology. However, this was a self-reporting study and the presence of an underlying disease could not be ruled out [29, 141]. More recent studies have demonstrated that the anti-LEDGF/p75 antibody is detected both in apparently healthy individuals and patients with a variety of non-rheumatic inflammatory conditions [37]. The increased frequency of LEDGF/p75 autoantibodies in apparent healthy individuals does not necessarily rule out disease since the immune system in these individuals may recognize an aberrant expression of this protein that could be associated with an undiagnosed pathological condition.

Our group previously reported that 22.3% of PCa patients from a Loma Linda University cohort had circulating serum antibodies that reacted with LEDGF/p75 either by ELISA or immunoblotting, compared to 6.7% of matched controls. The increase in the frequency of LEDGF/p75 autoantibodies in PCa patients is consistent with the elevated LEDGF/p75 expression in PCa tissues compared to normal adjacent tissue observed by our group [31, 36]. This observation of elevated frequencies of anti-LEDGF/p75 autoantibodies was later independently confirmed by two other groups [142-143], and is in agreement with the observation that altered LEDGF/p75 expression and function may contribute to tumor aggressive properties, which are discussed more extensively below [31-36, 59, 77-78, 83, 86-89, 120, 131, 144]. In a conflicting report, there were no detectable LEDGF/p75 autoantibodies in 40 cancer patient sera screened
by a chemiluminescence method, however, the cancer types were not disclosed [140]. We could speculate from all these studies that aberrant LEDGF/p75 overexpression, secretion, or cleavage during cell death might trigger autoantibodies under inflammatory conditions in certain genetically susceptible individuals.

Autoantibody reactivity to tumor associated autoantigens such as p90, survivin, and p53 has been used in experimental studies to complement prostate specific antigen (PSA) testing, which is currently unable to effectively distinguish between inflammatory, benign, and malignant conditions of the prostate leading to a significant number of unnecessary biopsies [145-149]. The inclusion of LEDGF/p75 into panels of TAAs for serum autoantibody profiling improved the predictive frequency in patients with PCa compared to the PSA blood tests [142-143]. The inherent function of the immune system to recognize and report changes in the expression or structure of a normal cellular protein expression provides a window into early changes that could lead to a disease state. This is leading to an increasing interest in TAAs and anti-TAA autoantibodies in cancer patients as potential diagnostic and prognostic tools, as well as reagents to develop novel immunotherapies [150].

**Role of LEDGF/p75 in Leukemia**

The role of LEDGF/p75 in cancer was first hinted in various early studies revealing that chromosomal translocations may result in fusion proteins involving this protein, with potentially altered transcription function [86, 117]. This role has now been well documented in studies showing that a fusion between NUP98 gene (chromosomal location: 11p15.5) and LEDGF/p75 (chromosomal location: 9p22.3) results in a NUP98-
LEDGF/p75 fusion gene with aberrant and malignant functions [86-91, 117-118]. The NUP98 gene encodes nucleoporin 98, a component of the nuclear pore complex that mediates nucleo-cytoplasmic transport of protein and mRNA [119]. It is involved in multiple rearrangements and fusions with more than 26 partner genes by translocations or inversions in hematological malignancies resulting in 5’-NUP98/3’-partner fusion genes [120-121]. The N-terminus of NUP98 contains Phe-Gly (FG) repeats that are known to interact with CREB binding protein (CBP) and its homologue p300, and act as oncogenic transactivation domains [122]. The NUP98-LEDGF/p75 fusion gene, likewise, involves the fusion of the C-terminus of LEDGF/p75 with the N-terminus of NUP98 caused by a rare but recurrent chromosomal translocation t(9;11)(p22;p15) that has been reported in four adult AML [86-87, 90, 117], one pediatric AML case [88], one chronic myeloid leukemia case [89], and one myelodysplastic syndrome (MDS) case [91]. All of the five AML cases reported with NUP98-LEDGF/p75 fusion are de novo, not related to therapy and have only t(9;11)(p22;p15) cytogenetic abnormality at diagnosis [90]. Among the seven reported cases of hematological malignancies with t(9;11)(p22;p15), only two were males, suggesting higher prevalence of NUP98-LEDGF/p75 fusion gene in females. This is consistent with reports of other NUP98 chimeras such as NUP98-HOXA and NUP98-TOP1 being more common in women [120, 123]. It is evident from the mortality associated with the seven cases that NUP98-LEDGF/p75 fusion gene is a poor prognostic marker associated with leukemia and MDS [90-91].

Although the molecular mechanism of AML/CML/MDS with t(9;11)(p22;p15) fusions is still unclear, it has been speculated that NUP98 fusion genes induce a pre-leukemic phase and that additional mutations in other cancer genes such as RAS are
required for progression to AML [91]. Available literature suggests different modes of action of NUP98 fusion with homeobox (eg. HOXA9, HOXD13) and non-homeobox (eg. TOP1, LEDGF/p75, DDX10) genes [124]. All the non-homeobox genes, including LEDGF/p75, were predicted to form “coiled-coils” which could lead to dimerization, oligomerization or the formation of multimeric complexes that enhance interaction with other transcription factors and co-factors [124]. It should be also noted that the NUP98-LEDGF/p75 fusion removes the N-terminal region of LEDGF/p75, implicated in regulating the transcriptional activities of this protein [125], resulting in a LEDGF/p75 variant with deregulated transcriptional activity and, consequently, increased pro-survival function in leukemic cells.

There have been several other reports implicating LEDGF/p75 in leukemia. Identification of tumor-associated antigens in chronic lymphocytic leukemia (CLL) by SEREX (serologic identification by recombinant expression cloning) technique yielded an antigen that was identified as LEDGF/p75 [126]. Transcript expression of LEDGF/p75 was also found to be upregulated in blasts from chemotherapy-resistant AML patients, and its ectopic overexpression protected leukemia cells against drug-induced apoptosis [32]. In a seminal study, Yokoyama and Cleary demonstrated that LEDGF/p75 tethers the menin-MLL transcription factor complex to chromatin to activate leukemogenesis [59]. This study also established LEDGF/p75 as a critical co-factor required for both promoting leukemic transformation and tumorigenic functions of MLL/menin complexes. Recently, Huang et al. analyzed the crystal structure of menin with an MLL protein 1 (MLL1)-LEDGF/p75 heterodimer and concluded that menin assembles a menin-MLL1-
LEDGF/p75 ternary complex to regulate gene transcription and promotes leukemogenesis [32].

Another study assessing the anticancer potential of marine compounds derived from benthic cyanobacteria in AML cells concluded that strain M44 was highly promising since its activity induced apoptosis in chemoresistant AML cells with enforced expression of LEDGF/p75, thus counteracting the protective effects of this protein [127]. The relevance of LEDGF/p75 in the development of leukemia has prompted researchers to explore targeting this protein as a therapeutic strategy [85, 128-130].

**Overexpression and Role of LEDGF/p75 in Solid Tumors**

Our group documented the upregulation of LEDGF/p75 in different cancer cell models compared to non-tumor cells, while its antagonistic alternative splice variant LEDGF/p52 is expressed at relatively low levels in cancer cells [26, 36, 63]. Our group was also the first to document the elevated LEDGF/p75 protein expression in clinical prostate tumor and benign prostatic hyperplasia tissues as compared to their corresponding normal tissues [36].

For several years, the only report of an immunohistochemical (IHC) LEDGF/p75 protein expression analysis in human tumor tissues was the one performed by our group in PCa [36]. Other reports had only examined upregulation of LEDGF/p75 transcript expression in leukemia, breast, and bladder cancers [32, 34]. To establish the upregulation of LEDGF/p75 in cancer, our group published a comprehensive report on the mRNA and protein expression of LEDGF/p75 in 21 major human tumor types, using cancer gene microarray databases, TissueScan Cancer Q-PCR array, and
immunohistochemical (IHC) analysis of tissue microarrays (TMAs) comprising 1735 tissue cores for this cross-platform analysis [31]. This study revealed selective LEDGF/p75 overexpression in human cancers, with statistically significant upregulation of both transcript and protein in prostate, colon, and thyroid malignancies. Bioinformatics analysis of PSIP1/LEDGFp75 transcript expression in the Oncomine database showed significant upregulation (tumor versus normal) in 15 of 17 tumor types. The TissueScan Cancer Q-PCR array revealed significantly elevated LEDGF/p75 mRNA expression (>1.5 fold) in 4 out of 8 tumor types, including prostate, colon, thyroid, and breast cancers. Significant overexpression of LEDGF/p75 protein was observed in prostate, colon, thyroid, liver and uterine tumors, relative to corresponding normal tissues when the IHC analysis was performed in TMAs. This upregulation was associated with younger age in patients with liver and thyroid tumors [31].

The role of LEDGF/p75 as a candidate oncoprotein in cancer cells has been further corroborated by reports of its elevated transcript expression in human breast and bladder cancers, and the observation that its ectopic overexpression in breast cancer cells protected against drug-induced lysosomal cell death [34]. Consistent with this, our group demonstrated that overexpression of LEDGF/p75 in PCa cells attenuated lysosomal cell death induced by stressors that trigger oxidative stress such as the anti-tumor drug DTX [33]. LEDGF/p75 was also shown to enhance the tumorigenic potential of HeLa cancer cells in murine xenograft models [34]. In ovarian tumors, lymphangiogenesis and angiogenesis was observed as a result of increased LEDGF/p75-dependent activation of VEGF-C [78]. It was also reported that LEDGF/75 overexpression increased tumorigenic characteristics in metastatic DU145 prostate cancer cells since its depletion
impaired the proliferative and invasive ability of these cells [83]. Leitz and colleagues [131] also demonstrated that LEDGF/p75 is overexpressed in human papilloma virus (HPV)-positive tumors and that downregulation of endogenous HPV18 E6/E7 gene expression repressed LEDGF/p75 expression in HeLa cells. Transduction of the HPV16 E6, E7 or E6/E7 genes resulted in LEDGF/p75 promoter activation and increased gene expression. Importantly, LEDGF/p75 was shown to be crucial for the survival of HPV-positive cancer cells after DNA damage by genotoxic agents [131].

**LEDGF/p75 Promotes Chemoresistance in Cancer Cells**

Despite the recent development of new chemotherapeutic drugs, many PCa patients eventually develop resistance to chemotherapy [15]. Several studies have implicated multiple mechanisms in the development of chemoresistance, such as the upregulation of pro-survival stress proteins, regulation of oxidative genes, induction of cytoprotective chaperones, and anti-apoptotic pathways [151]. Another important contributor to multi-drug resistance is increased expression of P-glycoprotein (P-gp), a member of the ABC superfamily of transporters. P-gp expression is upregulated in an inherent or acquired process, leading to ATP-dependent drug translocation across the cellular membrane [152]. This decreases the concentration of drugs inside the cell contributing to multi-drug resistance [153]. Despite ongoing studies to understand mechanisms of chemoresistance, we are still elucidating these pathways with the purpose of better predicting drug response and effectively treating advance disease [151-152].

Various studies suggest that LEDGF/p75 is a potential therapeutic target to circumvent cancer chemoresistance [32-34, 36, 83, 144]. Increased expression of
LEDGF/p75 has been associated with increased tumorigenic properties and chemoresistance in various cancers. For instance, Huang et al [32] reported increased mRNA levels of LEDGF/p75 in AML blast from patients that had developed chemoresistance. AML cells ectopically (plasmid-mediated) overexpressing LEDGF/p75 showed a significant increase in survival compared to cells expressing the empty vector when both cell lines where treated with the anti-leukemic drug daunorubicin or cAMP analogs. These results suggested that increased expression of LEDGF/p75 contributes to chemoresistance in AML [32].

Daugaard et al. [34] demonstrated that Hsp70-2 is a regulator of LEDGF/p75 expression in various cancer cell lines and that LEDGF/p75 knockdown with two different small inhibitory RNAs (siRNAs) resulted in increased caspase-independent cell death. The observed cell death was a result of lysosomal membrane permeabilization (LMP). Ectopic expression of LEDGF/p75 in MCF-7 cells resulted in protection against agents that increase LMP such as siramesine, etoposide, doxorubicin (DOXO), and TNF but not against the classical apoptotic-inducer staurosporine (STS) [34]. In a separate study, Daugaard et al. also reported that LEDGF/p75 is necessary for effective DNA double strand breaks repairs (DBSs) [144]. Osteosarcoma cells depleted of LEDGF/p75 were unable to effectively repair DNA DBSs when they were exposed to DNA damaging agents such as the drugs camptothecin and mytomycin, or ionizing radiation, resulting in decreased cancer cell survival [144].

The link between LEDGF/p75 overexpression in PCa and resistance to chemotherapy has also been explored in our laboratory. Our group demonstrated that the taxane drug DTX, the current FDA-approved standard of chemotherapy for the treatment
of advanced PCa, induces a caspase independent cell death through lysosomal destabilization and cathepsin B activation in PCa cells [33]. In that study, PC3 and RWPE-2 PCa cells with stable plasmid-mediated overexpression of LEDGF/p75 were found to be resistant to DTX as compared to cells transfected with an empty vector. These LEDGF/p75 overexpressing cells, when treated with DTX, exhibited stable lysosomes. However, LEDGF/p75 overexpression did not abrogate DTX inhibition of microtubule depolymerization or cell death induced by TNF-related apoptosis inducing ligand (TRAIL), suggesting that LEGDF/p75 may be a selective inhibitor of cell death, protective against LMP but not against mitotic catastrophe or apoptotic cell death [33].

Consistent with these results, we recently demonstrated that LEDGF/p75 overexpression in PCa cells promoted protection against caspase-independent necrotic cell death induced by tert-butyl hydrogen peroxide (TBHP), a potent inducer of cellular oxidative stress, possibly through the upregulation of antioxidant pathways and protective proteins such as ERp57/GRP58 [35]. We also showed that PCa cells grown in the presence of DTX and selected for their resistance to this drug, have high endogenous levels of this protein compared to the parental, drug-sensitive cell lines [35, 81]. As described in the next chapter, the studies conducted for this dissertation demonstrated that these cells overexpressing LEDGF/p75 were not only resistant to DTX but also exhibited resistance to other taxane drugs used in clinical settings such as PTX and CBZ.

**Targeting LEDGF/p75 and its Potential in Combinatorial Therapy**

When a patient develops PCa, androgen deprivation therapy (ADT) is an effective but temporary treatment alternative until tumors continue to grow without the presence of
androgens. At this stage, systemic therapy with different rounds of the chemotherapeutic taxane drug DTX, and more recently CBZ, are the only alternatives available to increase patient survival [154]. Combinatorial therapies such as DTX with anti-androgen drugs or other biologic agents, and even novel single agents are currently being studied with mixed results [155]. However, as PCa advances the tumors metastasize to the bones and critical organs, while developing resistance to therapy, including taxane-based chemotherapy [155]. In order to develop more effective therapeutic alternatives for advanced PCa, there is a need to understand the mechanisms underlying tumor cell survival - pre and post therapy [155].

Our team and others have demonstrated that LEDGF/p75 overexpression in different cancers types and its potential role in chemoresistance, making this protein an attractive target for therapeutic targeting in cancer patients [31-33, 59, 89]. Importantly, LEDGF/p75 is not essential for cell viability under normal, non-stressful growth conditions since cancer cell lines with stable knockdown of this protein have been successfully developed [131, 156-157]. *In vivo* studies have shown that depletion of this protein is not embryonically lethal given that *Psp1/LEDGF/p75−/−* knockout mice were born alive despite suffering from multiple skeletal malformations that resulted in increased perinatal mortality due to inability to nurse [100]. However, under stress conditions that include chemotherapy, LEDGF/p75 plays an important role in cell survival. This was highlighted by previous studies from our lab showing that overexpression of caspase cleavage fragments of LEDGF/p75 accelerated cell death in cancer cells only under stress conditions [26]. This makes this protein an attractive target
for the development of targeted therapeutic approaches in combination with cytotoxic
drugs such as DTX that elevate intracellular levels of oxidative stress [33].

The feasibility of targeting LEDGF/p75 in cancer cells has been demonstrated in
various studies utilizing siRNAs and small hairpin RNA (shRNA) to transiently and
stably knockdown its expression, respectively. Daugaard and colleagues utilized two
different non-overlapping siRNAs (designated L1 and L3) to study the effects of
LEDGF/p75 downregulation in various cancer cell lines [34]. They observed a decrease
in cellular density and an increase in the number of round and detached cells when
LEDGF/p75 expression was decreased in HeLa (cervical cancer), MCF-7 (breast cancer)
and U2OS (osteosarcoma) cells. Cells depleted of LEDGF/p75 exhibited an increase in
the volume of the acidic compartment, increased autophagosomes, and destabilization of
their lysosomal membranes. They concluded from these findings that LEDGF/p75
expression contributed to cell viability by stabilizing the lysosomal membranes [34].

In a more recent study, Daugaard and colleagues used siRNA to decrease the
expression of LEDGF/p75 in various human cancer cell lines and also in embryonic
fibroblasts (MEFs) derived from Psip1/LEDGF-knockout mice [144]. They demonstrated
that cells depleted of LEDGF/p75 were more susceptible to DNA-damaging agents such
as ionizing radiation, camptothecin and mitomycin. Knockdown of this protein impaired
the ability of the cells to repair DNA double-strand breaks. LEDGF/p75 knockdown also
inhibited the recruitment of CtIP, an important protein in the resection of DNA double-
strand breaks, to active chromatin sites, leading to DNA damage and cell death [144].

In another study published by Bhargavan et al. [83], siRNA was also used to
knockdown expression of LEDGF/p75 in DU145 prostate cancer cells and observe its
effects in various characteristics of tumor aggressiveness. In their study, LEDGF/p75 knockdown resulted in down-regulation of transactivation of the Hsp27 promoter, and decreased proliferation, migration, invasion and cell viability. Knockdown of LEDGF/p75 also reduced proliferation in normal epithelial prostate cells (PWR-1E) although not as significant as in the DU145 cells. These authors also observed that cell cycle progression was interrupted at the S-phase and G2–M phase in cells depleted of LEDGF/p75, associated with downregulation of the ERK1/2-Akt pathway. The tumor aggressive properties of these cells were re-established when LEDGF/p75 was ectopically re-expressed [83].

Our group utilized siRNA to knockdown the expression of LEDGF/p75 in PC3 and U2OS cells and demonstrated that LEDGF/p75 knockdown positively affects Hsp27 promoter transactivation by its interacting partner, the transcriptional factor MeCP2, possibly through a feedback loop [82]. As mentioned above, our group also utilized siRNA to knockdown the expression of LEDGF/p75 in PC3 cells to identify target genes of this protein [79]. Leitz et al. [131] utilized both shRNA and siRNAs to silence the expression of LEDGF/p75 in HPV18-positive (HeLa) and HPV16-positive (SiHa, CaSki) cells. They observed that 3 non-overlapping shRNAs targeting LEDGF/p75 reduced the clonogenic capacity of these cells. LEDGF/p75 depletion also reduced clonogenicity in HPV-negative cells regardless of their p53 status. In addition, silencing of LEDGF/p75 with siRNA sensitized cells to genotoxic agents such as hygromycin B, camptothecin (CPT), or 6 Gy γ-irradiation [131]. More recently, our group demonstrated that transient and stable downregulation of LEDGF/p75 in PC3 cells resulted in reduced transcript and protein expression of ERp57/GRP58, an oxidoreductase and chaperone of newly
synthetized glycoproteins [35]. Taken together, these studies demonstrate that LEDGF/p75 can be effectively targeted in cellular models to induce changes in cellular viability and tumorigenic properties.

**Repurposing HIV-Based LEDGF/p75 Inhibitors for Cancer Treatment**

LEDGF/p75 is widely recognized in the literature for its pivotal role in HIV-1 integration and replication [158]. Numerous studies have validated the integrase binding domain (IBD) in the C-terminal region of LEDGF/p75 as a binding site for the catalytic domain of HIV-integrase (IN), facilitating HIV integration and replication (Figure 5) [158]. Current efforts are focusing on the development of novel antiviral drugs that disrupt the interaction between HIV-IN and the IBD of LEDGF/p75 [159]. Recent studies reported the use of peptidomimetics and small molecule inhibitors (SMIs) that disrupt this interaction, decreasing HIV-IN binding activity and viral replication [160-161]. These studies point to LEDGF/p75 as a potentially druggable target, with implications for the treatment of HIV-AIDS and cancers overexpressing this protein.

As mentioned above, previous studies from our group demonstrated that cleavage of LEDGF/p75 by caspases during apoptotic cell death resulted in fragments with a truncated IBD incapable of promoting cancer cell survival under stress conditions, possibly due to the inability of these fragments of tethering transcription factor cargo to chromatin [26, 35, 63]. Moreover, our group also showed that overexpression of LEDGF/p52, which lacks the IBD, promoted apoptosis in cancer cells [63]. These early studies provided initial evidence for the important role of the IBD in cancer cell survival.
Figure 5. The IBD region of LEDGF/p75 facilitates viral infection by interacting with HIV-IN and tethering the pre-integration complex to the host’s chromatin (Adapted from Suzuki et al. 2011).
The feasibility of targeting LEDGF/p75, particularly its IBD region, in cancer has been recently demonstrated in the context of development novel treatments for MLL-fusion driven leukemias [130]. These are genetically diverse and characterized by a poor prognosis [129]. As mentioned previously, MLL forms a ternary complex with menin (MEN1) and LEDGF/p75 that targets specific genes, inducing their activation or repression leading to increased leukemic transformation [129]. Structural and biochemical studies revealed that the LEDGF/p75 IBD interacts with menin and MLL and that this interaction occurs via the same binding site of other interacting partners of LEDGF/p75 including PogZ and JPO2 [128]. This domain then plays multiple roles in different contexts: transcriptional and pro-survival activities presumably through interactions with transcription factors and other chromatin-associated proteins, facilitating the tethering of MLL fusion proteins to chromatin in leukemia, and facilitating HIV-1 integration and infection. Select SMIs designed to prevent HIV-1 IN and LEDGF/p75 complex formation through the IBD have shown antiviral properties without inhibiting IN enzymatic function [159, 162-163].

Recognizing the therapeutic potential of the multi-functional IBD domain of LEDGF/p75, involved in protein-protein interactions and pro-survival functions, there is currently a strong interest in developing different agents targeting this region. In one study, LEDGF/p75 interacting peptides known to inhibit the LEDGF/ p75–HIV-1 integrase interaction were shown to impair clonogenic growth of primary murine MLL fusion-expressing leukemic cells and reduce ectopic HoxA9 expression [128]. Another group used peptides derived from LEDGF/p75 to disrupt the menin/MLL-LEDGF/p75 complex and its subsequent pro-leukemogenic properties [85].
The development of targeted cancer therapy agents that destroy cancer cells by targeting oncogenic proteins or tumor-associated antigens (TAA) without affecting normal cells has significantly progressed during recent years [164]. Part of this effort includes the repurposing of agents that have been developed previously for the use in a different disease to be used adjunct to anti-cancer systemic therapeutic regimes, which could significantly improve clinical outcomes in chemoresistant cancers. For example, antiviral classes of compounds have been explored for their anti-cancer therapeutics against oncogenic kinases in a panel of cell lines including prostate, breast, and colon cancer [165]. Because of its dual role in HIV infection and cancer, LEDGF/p75 is an attractive target in anti-cancer therapy, with several classes of SMIs of the LEDGF/p75-IN interaction reported in the literature with baseline cytotoxicity data in cancer cells [160, 162-163, 166-168]. These inhibitors, originally designed to disrupt HIV integration, could potentially be repurposed for treatment of chemoresistant cancers in which LEDGF/p75 levels and activity are increased. An advantage of repurposing LEDGF/p75-IN inhibitors in anti-cancer therapy is that they are likely to target the C-terminal IBD, thus disrupting LEDGF/p75-protein interactions that are important for cancer cell survival under stress, which may include chemotherapeutic stress [130].

**Summary Highlights**

- Treatment options for CRPC are limited and patients often develop resistance to therapy resulting in disease progression and decreased survival.

- There is a critical need to identify and target novel oncoproteins that contribute to CRPC therapy resistance to reduce PCa mortality.
• LEDGF/p75 is an emerging stress oncoprotein that promotes cellular survival against a wide variety of environmental stressors, including oxidative stress, radiation, heat, serum starvation, and cytotoxic drugs.

• LEDGF/p75 interacts with different transcription factors and chromatin binding proteins to transactivate specific stress survival- and cancer-associated genes depending on the microenvironment, cellular context, and stress stimuli.

• LEDGF/p75 is overexpressed in several human cancers, including PCa, and promotes cancer cell aggressive properties such as cell proliferation, clonogenicity, migration, invasion, angiogenesis, tumor volume, and chemoresistance.

• Targeting LEDGF/p75 with RNA interference or repurposed HIV-based LEDGF/p75 inhibitors is a promising strategy to re-sensitize chemoresistant CRPC to taxane therapy, leading to increased patient survival and decrease in the racial disparity mortalities associated with CRPC.

Figure 6 illustrates our working model. Briefly, environmental stress caused by various factors such as treatment with taxane drugs results in elevated intracellular ROS, leading to increased LEDGF/p75 expression. Overexpressed and activated LEDGF/p75 then interacts with other co-transcription factors to upregulate the expression of stress survival proteins that contribute to aggressive cancer cell properties, including chemoresistance. Tissue overexpression of LEDGF/p75 or extracellular release of this protein may also lead to autoantibody elicitation in susceptible individuals.
Figure 6. LEDGF/p75 overexpression as a result of environmental stressors contributes to aggressive tumor properties.
Hypothesis and Purpose of Dissertation Work

While the role of LEDGF/p75 in HIV integration and infection has been thoroughly studied over the past 15 years, this protein has recently attracted significant attention in the field of cancer research due to its pro-survival functions, including increasing resistance to chemotherapy, in cancer cells. In a multidisciplinary effort, the knowledge obtained from studying viral integration and the availability of LEDGF/p75 targeting agents (both siRNAs and repurposed SMIs) could be potentially be applied to the clinic to address the problem of PCa chemoresistance and the lack of effective treatment options at later stages of the disease.

In this dissertation work, we explored the role of LEDGF/p75 in PCa chemoresistance, the mechanisms by which LEDGF/p75 might protect cells against taxane therapy, and the targeting of this protein in drug-resistant PCa cellular models using siRNAs and SMIs. The overall hypothesis of this study is that elevated LEDGF/p75 expression promotes selective chemoresistance in PCa by antagonizing non-apoptotic/caspase-independent cell death, and that targeting this protein can contribute to effectively overcome the observed drug-resistance. The results obtained from this study will add to current efforts to develop effective therapeutic options to reduce chemotherapy resistance in CRPC patients.
References


CHAPTER TWO

TARGETING THE STRESS ONCOPROTEIN LEDGF/P75 TO SENSITIZE
CHEMoresistant prostate cancer cells to taxanes

Abstract

Prostate cancer (PCa) is associated with chronic prostate inflammation resulting in activation of stress and pro-survival pathways that contribute to disease progression and chemoresistance. The stress oncoprotein lens epithelium-derived growth factor p75 (LEDGF/p75), also known as DFS70 autoantigen, promotes cellular survival against environmental stressors, including oxidative stress, radiation, and cytotoxic drugs. Furthermore, LEDGF/p75 overexpression in PCa and other cancers has been associated with features of tumor aggressiveness, including resistance to cell death and chemotherapy. We report here that the endogenous levels of LEDGF/p75 are upregulated in metastatic castration resistant prostate cancer (mCRPC) cells selected for resistance to the taxane drug docetaxel (DTX). These cells also showed resistance to the taxanes cabazitaxel (CBZ) and paclitaxel (PTX), but not to the classical inducer of apoptosis TRAIL. Silencing LEDGF/p75 effectively sensitized taxane-resistant PC3 and DU145 cells to DTX and CBZ, as evidenced by a significant decrease in their clonogenic potential. While TRAIL induced apoptotic blebbing, caspase-3 processing, and apoptotic LEDGF/p75 cleavage, which leads to its inactivation, in both taxane-resistant and -sensitive PC3 and DU145 cells, treatment with DTX and CBZ failed to robustly induce these signature apoptotic events. These observations suggested that taxanes induce both caspase-dependent and -independent cell death in mCRPC cells, and that maintaining the structural integrity of LEDGF/p75 is critical for its role in promoting taxane-resistance. Our results further establish LEDGF/p75 as a stress oncoprotein that plays an important role in taxane-resistance in mCRPC cells, possibly by antagonizing drug-induced caspase-independent cell death.
Introduction

Prostate cancer (PCa) represents a significant health burden in the United States since it is the most frequently diagnosed cancer in men and the second leading cause of male cancer deaths after lung cancer (1). The rates of PCa incidence and mortality are variable among different racial groups, with African American men presenting a disproportionately high incidence and mortality compared to other ethnic/racial groups [1, 2]. Chronic inflammation of the prostate leading to an augmented state of cellular oxidative stress and activation of stress survival pathways has been linked to PCa pathogenesis and resistance to therapy [3–7].

Lens Epithelium-Derived Growth Factor of 75kD (LEDGF/p75) has recently emerged as a stress oncoprotein that promotes cellular survival against many different environmental stressors, including oxidative stress, radiation, heat, serum starvation, and cytotoxic drugs [8–20]. Also known as PC4 and SFRS1 interacting protein (PSIP1), and dense fine speckled autoantigen of 70 kD (DFS70), this protein has attracted considerable attention due to its broad relevance to cancer, autoimmunity, eye diseases, and HIV-AIDS [14, 15]. LEDGF/p75 is the target of autoantibody responses in a subset of patients with PCa [14, 21], as well as in patients with diverse chronic inflammatory conditions and some apparently healthy individuals [14]. While early studies suggested that LEDGF/p75 was a growth factor critical for the proliferation of lens epithelial cells [8], subsequent studies have demonstrated that this protein is not a lens specific growth factor but rather a ubiquitous nuclear transcription co-activator with oncogenic functions that is activated during the cellular response to stress [14, 15].

Our group and others have shown that LEDGF/p75 is upregulated in PCa and in
other human cancer types, and that overexpression of this protein in cancer cells is associated with features of tumor aggressiveness, such as increased proliferation, resistance to cell death and therapy, invasion, migration, clonogenicity, angiogenesis, and tumor growth [11, 15–25]. In a previous study we reported that LEDGF/p75 overexpression in PCa cells promoted resistance against caspase-independent cell death induced through lysosomal membrane permeabilization (LMP) by the taxane drug docetaxel (DTX), the gold standard for advanced PCa chemotherapy [18]. These results were consistent with studies in other cancer cell types demonstrating that LEDGF/p75 overexpression promoted cellular protection against LMP-inducing drugs [19]. More recently, we provided evidence that LEDGF/p75 overexpression in PCa cells promotes protection against necrotic cell death induced by oxidative stress [20].

The mechanisms by which LEDGF/p75 promotes resistance to stress-induced cell death have not been fully elucidated, although available evidence suggests that this oncoprotein is upregulated or activated in response to environmental stressors [8-14, 17-20, 22, 24-25]. Acting as a transcription co-activator, it contributes to the transactivation of stress, antioxidant, and cancer-associated genes through interaction with transcription complexes involving RNA polymerase II, PC4 transcription factor, menin-MLL (mixed leukemia lineage), the MeCP2 transcription activator/repressor, and c-MYC-associated protein JPO2 [26–31]. LEDGF/p75 target genes include heat shock protein 27 (HSP27), oxidoreductase ERP57/ PDIA3/GRP58, cytoglobin (CYGB), peroxiredoxin 6 (PRDX6), involucrin, alcohol dehydrogenase, aldehyde dehydrogenase, αB-crystallin, gamma glutamylcysteine synthase, vascular endothelial growth factor C (VEGF-C), and interleukin 6 (IL-6) [12, 13, 20, 22, 23, 28, 32-41].
Recent evidence points to LEDGF/p75 as a promising druggable target for HIV and leukemia therapy [42–44]. In light of our previous demonstration that LEDGF/p75 overexpression in PCa cells promoted resistance to DTX [18], the present study was conducted to determine if targeting LEDGF/p75 in chemoresistant PCa cells would re-sensitize these cells to the clinically relevant taxane drugs DTX and cabazitaxel (CBZ), which are the first and second line cytotoxic chemotherapeutic drugs, respectively, approved by the Food and Drug Administration (FDA) for the treatment of metastatic castration-resistant prostate cancer (mCRPC) [45, 46]. In addition, since LEDGF/p75 promoted protection against DTX-induced lysosomal cell death and stress-induced caspase-independent cell death in PCa cells [18, 20], we explored if the protective functions of LEDGF/p75 are linked to the ability of DTX and CBZ to activate caspase-independent mechanisms of cell death in drug-resistant PCa pre-clinical models. This study represents the first step in the development of a multi-targeting approach involving LEDGF/p75 in combination with taxanes to re-sensitize chemoresistant mCRPC cells to therapy.

Results

**LEDGF/p75 is Overexpressed in DTX-Resistant DU145 and PC3 Cells**

We determined the expression of LEDGF/p75 in the DTX-resistant mCRPC cell lines DU145-DR and PC3-DR, compared to the drug sensitive parental DU145 and PC3 cells. These cells were developed to be resistant to DTX by selecting and expanding the surviving cells after successive treatments with increasing concentrations of the drug. We observed that the expression of LEDGF/p75 was significantly upregulated at the
transcript and protein level as DU145 and PC3 cells made the transition from chemosensitivity to chemoresistance (Figure 7). The DU145-DR cells displayed a significant 3.87 fold increase in LEDGF/p75 transcript expression compared to the parental DU145 cells (Figure 7A, left panel). To determine if the increase in transcript expression correlated with increased protein expression, we collected total lysates from DU145 and DU145-DR cells and performed immunoblotting using an antibody specific for LEDGF/p75. We observed that LEDGF/p75 was robustly expressed in the DU145-DR cells compared to the parental cells (Figure 7B, left panel). To further confirm these findings, we then proceeded to analyze LEDGF/p75 expression by immunofluorescence microscopy using a well-characterized human autoantibody against this protein [47]. Acquiring the images under exactly the same parameters, we observed that the fluorescence intensity of nuclear dense fine speckles, corresponding to LEDGF/p75 staining [28, 47], in the DU145-DR cells was higher compared to the intensity in the chemosensitive DU145 cells (Figure 7C, left panel).

The same experimental procedures were performed to assess LEDGF/p75 expression in the PC3 and PC3-DR cells. When we compared the LEDGF/p75 transcript expression in these two cell lines, we observed a significant 3.60 fold increase in the transcript levels in PC3-DR compared to the sensitive PC3 cells (Figure 7A, right panel). As in DU145-DR cells, there was a robust increase in LEDGF/p75 protein expression in the PC3-DR cells compared to the parental PC3 cells (Figure 7B, right panel). Also, similar to DU145-DR cells, the fluorescence intensity of LEDGF/p75 staining in PC3-DR cells was higher when compared under identical imaging conditions to the PC3 cells (Figure 7C, right panel). Taken together, these findings showed higher endogenous
expression of LEDGF/p75 in DTX-resistant cells at both the transcript and protein levels compared to their drug-sensitive parental cells.
A

** $p < 0.01$

Fold change in LEDGF/p75 mRNA expression

DU145 | DU145-DR

PC3 | PC3-DR

B

LEDGF/p75 (75-kDa)

β-Actin (42-kDa)

C

DU145

DU145-DR

PC3

PC3-DR
Figure 7. LEDGF/p75 is overexpressed in DTX-resistant DU145 and PC3 cells. LEDGF/p75 transcript levels were quantified using mRNA isolated from DU145 and DU145-DR cells by qPCR in at least three independent experiments (A, left panel). Statistical significance was determined in comparison to control DU145 cells using Student’s t-test (** p<0.01). LEDGF/p75 protein expression was evaluated in lysates from DU145 and DU145-DR cells by immunoblotting using a rabbit anti-LEDGF/p75 antibody that specifically detects this protein at 75kDa (B, left panel). β-actin was used as loading control. Fluorescence intensity of nuclear dense fine speckles characteristic of LEDGF/p75 was evaluated by indirect immunofluorescence microscopy in DU145 and DU145-DR cells, using a human anti-LEDGF/p75 autoantibody (C, left panel). LEDGF/p75 transcript levels were also quantified using mRNA isolated from PC3 and PC3-DR cells by qPCR in at least three independent experiments (A, right panel). P values were determined in comparison to control PC3 cells using Student’s t-test (**p<0.01). LEDGF/p75 protein expression was evaluated in lysates from PC3 and PC3-DR cells by immunoblotting using a rabbit anti-LEDGF/p75 antibody (B, right panel). β-actin was used as loading control. Fluorescence intensity of nuclear dense fine speckles characteristic of LEDGF/p75 was evaluated by indirect immunofluorescence microscopy in PC3 and PC3-DR cells, using a human anti-LEDGF/p75 autoantibody (C, right panel).
DU145-DR and PC3-DR Cells are Resistant to Multiple Taxanes but not to TRAIL

We then investigated if the DTX-resistant PCa cells, which showed endogenous overexpression of LEDGF/p75, were selectively resistant to DTX or also showed multi-drug resistance, particularly to other taxanes such as CBZ and paclitaxel/taxol (PTX). Currently, CBZ is the second-line cytotoxic chemotherapeutic drug available for advanced PCa patients that develop resistance to DTX [45, 46]. We also included in our analysis PTX, the original taxane and parent drug of both DTX and CBZ. Although not currently used for clinical treatment of advanced PCa, PTX is commonly used in the treatment of other tumor types [48], and it was therefore important to determine if DTX-resistant cancer cells overexpressing LEDGF/p75 also promote resistance to this parental taxane. We also treated our chemosensitive and chemoresistant PCa cells with tumor necrosis factor related apoptosis inducing ligand (TRAIL), an inducer of caspase-dependent apoptosis. In previous studies we observed that while ectopic LEDGF/p75 overexpression promoted protection against stressors that induced caspase-independent cell death such as DTX and tert-butyl hydroperoxide (TBHP), it did not confer protection against classical inducers of caspase-dependent cell death such as TRAIL and staurosporine (STS) [18, 20]. Therefore, we sought to reproduce these observations in chemoresistant PCa cells naturally overexpressing LEDGF/p75 after selection.

For these studies, we treated DU145-DR cells and DU145 cells with increasing concentrations of DTX, PTX, or CBZ for up to 72 hr, and TRAIL for up to 24 hr (Figure 8A) and determined the approximate drugs’ half-maximal effective concentrations (EC50). We observed higher viability in DU145-DR cells treated with DTX (EC50 ≈ 200 nM), and PTX (EC50 ≈ 300 nM), compared to DU145 cells, which showed EC50s of 10
nM and 20 nM, respectively. In the dose response curves, a statistically significant difference in the percentage of surviving cells could be observed at all concentrations (range 10-1000 nM). Interestingly, when we examined the difference in the overall survival between DU145-DR and DU145 cells treated with CBZ, there was a trend toward higher survival in the DU145-DR cells, with EC50 values of 11 nM in the DU145-DR cells and 2 nM in the DU145 cells. For CBZ, there was a statistically significant difference in survival at concentrations above 10 nM. We did not observe a difference in survival when DTX-resistant and -sensitive cell lines were treated with increasing concentrations of TRAIL. Both cell lines showed high sensitivity to low concentrations of this cell death ligand (10 ng/ml).

Similar experiments with PC3-DR and PC3 cells revealed a statistically significant difference in the percentage of surviving PC3-DR cells compared to PC3 cells after exposure to DTX, with EC50s of 100 nM and 10 nM, respectively (Figure 8B). We also observed a statistically significant increased survival in PC3-DR cells exposed to PTX, with an EC50 of 1000 nM, compared to an EC50 of 100 nM for PC3 cells. Consistent with results obtained with the DU145-DR and DU145 cells, there was an overall trend in the difference in survival between PC3-DR cells and PC3 cells after treatment with CBZ. We obtained EC50s of 50 nM for PC3-DR and 5 nM for PC3 cells, with statistically significant differences at CBZ concentrations below 1 nM. Like in DU145 cells, we did not observe any differences when PC3-DR and PC3 cells were treated with TRAIL.

We then proceeded to examine the morphology of the cells under the different treatment conditions (Figure 8C). We observed that DTX-resistant cells had a
relatively normal morphology with fewer floating cells and features of cell death, compared to the sensitive cell lines which clearly showed increased cell death when treated with DTX, PTX, or CBZ at the low pharmacological concentrations of 10 nM, 1 µM, and 10 nM, respectively. Robust apoptotic cell death could be observed upon treatment with TRAIL. In summary, there was increased cell survival in the DTX-resistant cell lines, which express high endogenous levels of LEDGF/p75, during treatment with increasing concentrations of the different taxanes. However, none of the cell lines showed resistance to TRAIL.
Figure 8. DU145-DR and PC3-DR cells are resistant to multiple taxanes but not to TRAIL. **A.** and **B.** Assessment of cell viability as measured by MTT assay in DU145 (red diamonds), DU145-DR (blue squares), PC3 (red triangles), and PC3-DR (crosses) cells treated with increasing concentrations of DTX (10nM, 50nM, 100nM, 500nM, and 1000nM), PTX (10nM, 50nM, 100nM, 500nM and 1000nM), CBZ (0.1 nM, 1nM, 10nM, 50nM and 100nM) for up to 72 hr, and TRAIL (10ng/ml, 20ng/ml, 50ng/ml, 80ng/ml and 100ng/ml) for 24 hr. Each graph represents the average of at least three different experiments in triplicates normalized to untreated controls. Standard error of the mean (SEM) was calculated. Statistical significance was determined by comparing the values for each drug concentration in the DTX-sensitive DU145 and PC3 cells versus the DTX-resistant DU145-DR and PC3-DR cells, respectively, using Student’s t-test (*p<0.05). **C.** Morphology of drug-sensitive and -resistant cells after treatment with DTX, PTX, CBZ, or TRAIL, visualized by Hofmann Modulation Contrast microscopy. Scale bar set at 20 µM.
LEDGF/p75 Depletion Sensitizes DTX-resistant PCa Cells to Clinically Relevant Taxanes

Given that the transition from taxane sensitivity to resistance in PCa cells involves the upregulation of several survival pathways [49, 50], it was necessary to establish the contribution of LEDGF/p75 to the observed taxane resistance in DU145-DR and PC3-DR cells. For these experiments, we transiently knocked down LEDGF/p75 in our drug-resistant models using small inhibitory RNAs (siRNAs) specific for this protein [20, 33]. We sought to determine if LEDGF/p75 knockdown alone decreased the clonogenic potential of taxane-resistant cells, and if its combination with drug treatment further sensitized the cells to taxane chemotherapy. We chose clonogenic assays for these experiments because they could clearly show cellular sensitization to the treatments over time by decrease in colony formation. The spatial constraints (96 well plates) that we encountered in short-term MTT viability assays did not permit to assess the long-term effects of drugs on cell growth. Colony formation assays have been widely used to determine the effects of LEDGF/p75 knockdown on its tumorigenic properties as well as its ability to promote resistance to non-taxane drugs in non-PCa cell models [19, 23, 25, 27, 51].

Transient knockdown of LEDGF/p75 in DU145-DR and PC3-DR cells led to robust depletion of the protein compared to cells transfected with scrambled duplex siRNA (siSD) control (Figure 9A and 9A). LEDGF/p75 depletion was still robust 96 hr post-transfection in both cell lines, indicating that it was stable at the time the cells were treated with the DTX or CBZ during the initial hours of clonogenic growth (Figure 9A and 9A). We focused on DTX and CBZ on these and subsequent experiments because
they are the first and second line chemotherapeutic drugs, respectively, currently used for the clinical treatment of mCRPC.

LEDGF/p75 depletion alone in DU145-DR cells in the absence of taxanes significantly reduced colony formation by 50.7% compared to untreated siSD control cells (Figure 9B, 9C, 9D). On the other hand, treatment with DTX alone, without LEDGF/p75 depletion, significantly reduced colony formation by 35% and 55.3% at concentrations of 50 nM and 100 nM, respectively, compared to the untreated siSD control cells (Figure 9C), indicating an EC50 for DTX alone of approximately 100 nM. However, the combination of LEDGF/p75 depletion plus 50 nM DTX or 100 nM DTX reduced colony formation by 75.2% and 83.4%, respectively, when compared to untreated SD-control cells (Figure 9C), and by 49.7% and 66.4%, respectively, when compared to LEDGF/p75 depletion alone (Figure 9D). These results indicated that the combination of DTX plus LEDGF/p75 depletion chemosensitized the cells, with EC50 < 50 nM, compared to untreated cells.

In the case of CBZ, this drug alone, without LEDGF/p75 depletion, significantly reduced colony formation in DU145-DR cells by 14.2% and 41.8% at concentrations of 0.1 nM and 1 nM, respectively, when compared to untreated siSD-control cells (Figure 9B, 9C), indicating an EC50 above 1 nM. However, the combination of LEDGF/p75 depletion plus 0.1 nM CBZ or 1 nM CBZ reduced colony formation by 66.1% and 83.8%, respectively, when compared to untreated siSD control cells (Figure 9C), and by 31.2% and 67.1%, when compared to LEDGF/p75 depletion alone (Figure 9D). These results indicated that the combination of CBZ plus LEDGF/p75 depletion chemosensitized the cells, with EC50 < 0.1 nM, compared to untreated cells.
DU145-DR cells transfected with siSD control oligos or si-LEDGF/p75 were equally sensitive to 100 ng/ml TRAIL, a concentration used in previous studies to efficiently induce caspase-dependent apoptosis [18, 21] and which did not yield any colonies (Figure 9B, 9C, 9D). These results were consistent with results shown in Figure 2 in which DU145 cells, both DTX sensitive resistant, were equally sensitive to a wide range of TRAIL concentrations (10 ng/ml-100 ng/ml).

We then performed similar studies to determine the effects of transient LEDGF/p75 depletion in PC3-DR cells, with and without drug treatment. We observed a significant decrease (24.4%) in the clonogenicity of PC3-DR cells after LEDGF/p75 depletion compared to the siSD control cells, although the effect was not as robust as in the DU145-DR cells (Figure 10B, 10C). Treatment with DTX alone, without LEDGF/p75 depletion, reduced PC3-DR colony formation by 10.4% and 18.6% at concentrations of 50 nM and 100 nM, respectively, compared to the untreated siSD control cells (Figure 10C), indicating an EC50 well above 100 nM. However, the combination of LEDGF/p75 depletion plus 50 nM DTX or 100 nM DTX reduced colony formation in the PC3-DR cells by 46.7% and 57.9%, respectively, when compared to untreated SD-control cells (Figure 10C), and by 29.5% and 44.4%, respectively, when compared to LEDGF/p75 depletion alone (Figure 10D). These results indicated that the combination of DTX plus LEDGF/p75 depletion chemosensitized the cells, with an EC50 between 50 and 100 nM, compared to untreated cells.

Treatment of PC3-DR cells with CBZ alone, without LEDGF/p75 depletion, significantly reduced clonogenicity by 7.7% and 24.1% at concentrations of 0.1 nM and 1 nM, respectively, when compared to untreated siSD-control cells (Figure 10C), indicating
an EC50 well above 1 nM. However, the combination of LEDGF/p75 depletion plus 0.1 nM CBZ or 1 nM CBZ reduced PC3-DR colony formation by 36.2% and 54.2%, respectively, when compared to untreated siSD control cells (Figure 10B), and by 15.6% and 39.4%, when compared to LEDGF/p75 depletion alone (Figure 10D). These results indicated that the combination of CBZ plus LEDGF/p75 depletion chemosensitized the cells, with EC50 < 1 nM, compared to untreated cells.

Like in DU145-DR cells, we also observed that PC3-DR cells transfected with siSD control oligos or si- LEDGF/p75 were equally sensitive to 100 ng/ml TRAIL. Taken together, these results showed that LEDGF/p75 depletion in DU145-DR and PC3-DR cells significantly diminished their clonogenicity, an effect that was enhanced in combination with taxanes.
Figure 9. Transient knockdown of LEDGF/p75 sensitizes DU145-DR cells to clinically relevant taxanes DTX and CBZ. 

A. LEDGF/p75 knockdown was confirmed by immunoblotting using a rabbit anti-LEDGF/p75 antibody in DU145-DR cells transfected with si-LEDGF/p75 oligos as compared to cells transfected with the siSD control oligos.

B. Representative images of colony formation assay plates showing a decrease in clonogenicity in DU145-DR cells with LEDGF/p75 depletion compared to siSD control cells, in the presence and absence of drugs. Colonies were counted after 12 days of treatment.

C. Bar graph showing quantification of DU145-DR colonies. Each bar represents the average of colonies counted in at least three independent experiments. SEM was calculated.

D. Bar graph showing the percent clonogenicity in DU145-DR cells with LEDGF/p75 depletion, in the presence and absence of drug treatment, compared to untreated cells. Data was derived from the bar graph shown in panel C. SEM was calculated. Statistical significance was determined by comparing the values for cells transfected with siSD control oligos vs cells with LEDGF/p75 knockdown.
Figure 10. Transient knockdown of LEDGF/p75 sensitizes PC3-DR cells to clinically relevant taxanes DTX and CBZ. A. LEDGF/p75 knockdown was confirmed by immunoblotting using a rabbit anti-LEDGF/p75 antibody in PC3-DR cells transfected with si-LEDGF/p75 oligos as compared to cells transfected with the siSD control oligos. B. Representative images of colony formation assay plates showing a decrease in clonogenicity in PC3-DR cells with LEDGF/p75 depletion compared to siSD control cells, in the presence and absence of drugs. Colonies were counted after 12 days of treatment. C. Bar graph showing quantification of PC3-DR colonies. Each bar represents the average of colonies counted in at least three independent experiments. SEM was calculated. D. Bar graph showing the percent clonogenicity in PC3-DR cells with LEDGF/p75 depletion, in the presence and absence of drugs, compared to untreated cells. Data was derived from the bar graph shown in panel C. SEM was calculated. Statistical significance was determined by comparing the values for cells transfected with siSD control oligos vs cells with LEDGF/p75 knockdown, in the presence or absence of drugs, using Student’s t-test (*p<0.05).
**LEDGF/p75 Retains Structural Integrity During Taxane-Induced Cell Death but is Robustly Cleaved During TRAIL-Induced Apoptosis**

We showed previously that PCa cells with ectopic overexpression of LEDGF/p75 were more resistant to DTX-induced lysosomal cell death and to oxidative stress-induced necrosis, but not to classical apoptosis inducers such as TRAIL and staurosporine (STS) [18, 20]. In addition, studies from our group showed that during caspase-dependent cell death triggered by classical apoptosis inducers (e.g., Fas, TRAIL, STS, etoposide), LEDGF/p75 is cleaved by caspases-3 and -7, generating various cleavage fragments, including a signature fragment of 65 kD that lacks pro-survival activity and exacerbates cell death in the presence of stress [11, 20–21]. We also demonstrated that this protein has a short splice variant, LEDGF/p52, which induces apoptosis when ectopically overexpressed leading to LEDGF/p75 cleavage and impaired ability to transactivate stress survival genes [52]. Together, these observations suggested that the stress protective effects of LEDGF/p75 are more relevant in the context of cellular resistance to stress-induced caspase-independent cell death, where lack of caspase activation results in preservation of LEDGF/p75 structural integrity, which is essential for its transcriptional and stress survival functions [11, 32, 52].

In light of these previous observations and the observed contribution of LEDGF/p75 to cellular resistance to taxanes but not TRAIL, we designed experiments to determine if the structural integrity of LEDGF/p75 is preserved during taxane-induced cell death. For these studies, we treated DTX-sensitive and -resistant DU145 and PC3 cells with concentrations of DTX (100 nM), CBZ (100 nM), or TRAIL (100 ng/ ml) that caused cell death in the previous experiments (Figures 8-10). We first examined by
Western blotting if there was caspase-3 processing under these treatments, which would be indicative of activation of caspase-dependent apoptosis, using an antibody that specifically recognizes cleaved caspase-3 (Figure 11A). We observed the appearance of strong bands corresponding to cleaved caspase-3 fragment in lysates from both DTX-sensitive and -resistant cells treated with TRAIL. However, we did not detect robust processing of caspase-3 in DU145 or PC3 cells, both sensitive and resistant, 72 hr after exposure to 100 nM DTX, in spite of observing significant cell death at this concentration and time point, particularly in the sensitive cells, in previous experiments (Figure 8). Overexposure of chemiluminescent blots to film did not result in increased detection of caspase-3 cleavage in cells treated with DTX or CBZ (data not shown). There was a slight detection of cleaved caspase-3 in DU145 cells treated with 100 nM CBZ (Figure 11A), in spite of the extensive loss of cell survival observed at this concentration in previous experiments (Figure 8). These experiments suggested that while TRAIL clearly induces caspase-dependent cell death in these cell lines, both sensitive and resistant, DTX and CBZ did not induce a comparatively robust caspase-3 processing and activation at the relatively high pharmacological concentrations used.

We then proceeded to determine if LEDGF/p75 was cleaved during the same experimental conditions. As expected, we observed robust cleavage of LEDGF/p75 into its signature apoptotic 65 kD fragment in DU145 and PC3 cells, both sensitive and resistant, treated with TRAIL (Figure 11B). This fragment was detected using human anti-LEDGF/p75 autoantibodies, which recognize a C-terminal autoepitope region that is preserved in the apoptotic cleavage fragments [11, 20]. However, LEDGF/p75 was minimally cleaved during the 72 hr treatment with 100 nM DTX in all cell lines (Figure
Although cleavage was more visible, albeit still weak, in cells treated with 100 nM CBZ, it did not achieve the robustness of the cleavage induced by TRAIL (Figure 11B). These results were consistent with the observed minimal processing of caspase-3 during taxane-induced cell death, with robust processing during TRAIL-induced apoptosis (Figure 11A).

In light of these results we proceeded to examine closely the morphology of DU145 and PC3 cells, both DTX-sensitive and -resistant, treated with 100 nM DTX, 100 nM CBZ, or 100 ng/ml TRAIL to assess the features of cell death (Figure 11C). The TRAIL-treated cells exhibited the classical apoptotic morphology characterized by extensive blebbing and shrinkage. By contrast, cells exposed to DTX and CBZ exhibited rounding and floating, consistent with mitotic arrest and catastrophe, as well as cells that appeared to be swollen and with breakage of the cell membrane. While there were cells displaying apoptotic blebbing, this feature was not as prominent in the taxane-treated cells as in the cells treated with TRAIL. These results are consistent with the observation of robust caspase-3 processing and LEDGF/p75 cleavage during TRAIL-induced cell death (Figure 11A) but not during taxane treatment (Figure 11B).
Figure 11. Caspase-3 processing and LEDGF/p75 cleavage in mCRPC cells treated with taxanes and TRAIL. A. Caspase-3 processing was detected by immunoblotting using an antibody specific for its large subunit (17/19 kD). Whole lysates were obtained from DU145, DU145-DR, PC3 and PC3-DR cells after treatment with DTX or CBZ (100nM) for 24, 48 and 72 hr, or TRAIL (100ng/ml) for 6, 12, and 24 hr. Untreated cells were used as controls. β-actin was used as loading control. B. LEDGF/p75 cleavage was assessed using a human anti-LEDGF/p75 autoantibody that specifically detects this protein (75 kD) and its main apoptotic cleavage fragment (65 kD). Whole lysates were obtained from cells after treatment with DTX or CBZ (100nM) for 24, 48 and 72 hr, or TRAIL (100ng/ml) for 6, 12, and 24 hr. Untreated cells were used as controls. β-actin was used as loading control. C. Cell morphology was assessed by Hofmann Modulation Contrast microscopy to visualize features of cell death after drug treatment. Scale bar set at 20 µM.
LEDGF/p75 Depletion Does Not Influence the Expression of the Multidrug Resistance Protein P-glycoprotein in DTX-Resistant PCa Cells

The molecular mechanisms by which LEDGF/p75 promotes taxane resistance are relatively unknown, although they are likely linked to its ability to transcriptionally co-activate stress survival genes. Given the established role of multidrug resistance or transporter proteins such as P-glycoprotein (P-gp, also known as ABCB1 or MDR1) in PCa chemoresistance [53, 54], we speculated that LEDGF/p75 might upregulate this protein in taxane resistant cells. For these experiments, we first compared the expression of P-gp in DU145-DR and PC3-DR cells to the drug-sensitive, parental DU145 and PC3 cells. Consistent with its role in chemoresistance, P-gp was not expressed in the sensitive cell lines but was highly expressed in the drug-resistant cells (Figure 12A). We then determined if transient LEDGF/p75 depletion (up to 72h) in the taxane resistant cells led to downregulation of P-gp (Figure 12B). Our results indicated that LEDGF/p75 depletion had no effect on P-gp expression levels in DU145-DR and PC3-DR, suggesting that P-gp is not a target gene of this stress transcription co-activator.
Figure 12. LEDGF/p75 depletion in DTX-resistant cells does not lead to downregulation of P-glycoprotein. A. Immunoblots showing increased P-glycoprotein (P-gp) expression in DU145-DR and PC3-DR cells compared to the DTX sensitive, parental cell lines. B. Immunoblots showing that transient depletion (72 hr) of LEDGF/p75 in DU145-DR and PC3-DR does not attenuate P-gp expression. β-actin was used as loading control.
Discussion

Our understanding of mechanisms underlying mCRPC has improved the outcomes for the management of this disease, with new therapeutic regimens that include sipuleucel-T, denosumab, abiraterone acetate, enzalutamide, and taxane therapy [55]. Unfortunately, despite these advances and overall increase in patient survival, mCRPC is still a challenging disease to manage, with most patients dying within three years of diagnosis due to therapy resistance, particularly to taxanes [49, 50, 53, 55-58]. The goal of the present study was to further establish the role of LEDGF/p75 in PCa taxane resistance and its potential as a novel therapeutic target for overcoming this resistance.

Our group and others have provided evidence for the role of this protein in promoting tumor aggressive properties and chemoresistance in various cancer types [11, 17-20, 22-27]. We reported previously that stable overexpression of LEDGF/p75 in PC3 cells attenuated DTX-induced caspase-independent cell death caused by LMP, as well as oxidative stress-induced necrosis, but not to the apoptosis inducers TRAIL and STS [18, 20]. Given its role as a stress transcription coactivator, LEDGF/p75 activation by cancer cells is likely to counter rapid increases in oxidative stress that might overwhelm cellular antioxidant defense mechanisms and induce DNA damage and LMP.

In this study we used mCRPC cellular models (PC3 and DU145) that were selected over time for their acquired resistance to DTX. While multiple mechanisms of taxane- resistance likely operate in these cells, we focused on LEDGF/p75 given our previous observations linking its ectopic overexpression to DTX resistance in mCRPC cells [18]. Our results clearly demonstrated the upregulation of endogenous LEDGF/p75 in DU145-DR and PC3-DR cells. This upregulation did not appear to occur during short-
term exposure to DTX because cells treated with increasing concentrations of DTX for up to 48 hr did not show LEDGF/p75 upregulation (data not shown). However, as we selected chemoresistant clones after weeks of exposure to increasing concentrations of DTX, we began to detect elevated endogenous LEDGF/p75 levels, suggesting that this stress protein contributes to the selection of surviving cells in the presence of DTX.

Our results also showed that DTX-resistant DU145 and PC3 cells displayed increased resistance to PTX and CBZ, compared to sensitive cells, at a wide range of pharmacological concentrations. However, both DTX-sensitive and -resistant cell lines showed robust sensitivity to TRAIL. These results suggested that the mechanisms of DTX resistance operating in these cells could also apply to PTX and CTX resistance, but not to TRAIL. The exquisite sensitivity of taxane-resistant cells to TRAIL suggests that apoptosis induction via death receptor signaling could be used to bypass the cellular protective functions of LEDGF/p75 and other survival proteins that are susceptible to caspase-mediated cleavage and inactivation. Unfortunately, efforts to target TRAIL receptors in clinical trials have been tempered by increased toxicity to cancer patients, most likely due to the high levels of these receptors in normal tissues [59].

Recent efforts to target LEDGF/p75 in the context of HIV-AIDS and leukemia have provided “proof-of-principle” that this protein is a druggable molecular target [27, 43, 44, 51, 60]. We reasoned that if LEDGF/p75 upregulation occurs during development of taxane chemoresistance, then targeting this protein in pre-clinical mCRPC models in combination with taxanes would sensitize sub-populations of resistant tumor cells to these drugs. Our results revealed that transient LEDGF/p75 depletion by itself, without drug treatment, attenuated the clonogenicity of both DTX-sensitive and -
resistant PC3 and DU145 cells, consistent with results from other groups using other tumor cell models [19, 25]. It should be noted, however, that LEDGF/p75 is not essential for cell viability under normal growth conditions since cancer cell lines with stable knockdown of this protein have been effectively developed [20, 28]. Also, PSIP1/LEDGF/p75−/− knockout mice were viable despite suffering from multiple skeletal malformations leading to increased perinatal mortality [61]. However, under stress conditions, LEDGF/p75 plays a key role in enhancing cell survival [13, 14].

Our results also showed that LEDGF/p75 depletion in combination with DTX or CBZ significantly decreased the clonogenic potential of both DU145-DR and PC3-DR cells, particularly at higher, albeit still pharmacological, drug concentrations. Given the limited range of taxane concentrations used in our clonogenic assays, it was not possible to determine with precision the exact EC50 values for drug-resistant DU145 and PC3 cells treated with either DTX or CBZ, with or without LEDGF/p75 depletion. While our results suggest a possible additive effect of the combinatorial treatment of taxanes plus LEDGF/p75 depletion, we cannot rule out the possibility that this combination acts synergistically to re-sensitize resistant cells. This could be explored in future studies by applying the Chou Talalay statistical method [62] to results from experiments in which a broad range of concentrations of both taxanes and small molecule inhibitors of LEDGF/p75 are combined. Our data also indicated that the anti-survival effects of LEDGF/p75 silencing was more pronounced in DU145-DR cells, consistent with the previous observation that LEDGF/p75 silencing in DU145 cells impairs their aggressive properties [23].

LEDGF/p75 silencing did not completely sensitize resistant cells to DTX and
CBZ, most likely due to the contribution of other independent mechanisms, possibly involving clusterin and P-gp drug transporter, to taxane resistance [49, 50, 53, 54]. Indeed, our results demonstrated that LEDGF/p75 depletion does not downregulate P-gp in taxane-resistant cells, suggesting that these two proteins act independently of each other. We cannot rule out, however, that LEDGF/p75 may transcriptionally activate P-gp in resistant cells but the cellular stability of this drug transporter may not make it susceptible to downregulation in response to LEDGF/ p75 depletion. Interestingly, a previous study showed that while P-gp is dramatically upregulated in several chemoresistant PCa cell lines, its inhibition reversed the resistant phenotype on a cell-line dependent manner, which would be consistent with the notion that multiple mechanisms of drug resistance may be activated in prostate tumor cells in response to chemotherapy [54]. Identifying resistance mechanisms independent of P-gp is therefore important since targeting this drug- transporter has not been highly successful because of the complexity of toxicity, adverse side effects, and altered pharmacokinetics encountered in studies [63].

Maintaining the structural integrity of LEDGF/ p75, particularly its C-terminal domain, is essential for its transcriptional and stress survival functions [11, 32, 52]. During apoptosis, caspase-3 mediated cleavage removes the extreme N-terminal and C-terminal regions of LEDGF/p75, abrogating its stress survival functions [11]. This could explain why LEDGF/ p75 overexpression in cancer cells typically does not confer protection against insults that robustly activate caspase-3, such as TRAIL and STS, which result in LEDGF/p75 cleavage and inactivation, but does protect against insults that induce LMP and even necrotic cell death, which leave the protein relatively intact [18,
While several reports have underscored the anti-apoptotic role of LEDGF/p75 in cancer cells, the exact mode of cell death, and the possibility that caspase-dependent and caspase-independent pathways operate in parallel under the experimental conditions used, have not been fully characterized in these studies [17, 23, 24, 65]. Nevertheless, it is plausible that LEDGF/p75 could promote protection against apoptosis if this mode of cell death occurs downstream of events antagonized by this protein such as DNA damage and LMP [18-19, 24, 64].

Our observation that DTX and CBZ did not induce robust caspase-3 processing and LEDGF/p75 cleavage at relatively high pharmacological plasma concentrations that induce cell death (100 nM), suggests that insufficient apoptosis induced by taxanes may lead to drug resistance by preserving the structural integrity of LEDGF/p75 and other stress survival proteins that otherwise would be cleaved and inactivated during apoptotic caspase-3 activation (Figure 7). In a previous study, we showed that caspase-3 activity and LEDGF/p75 cleavage could be induced in PC3 cells, albeit not robustly, at micromolar concentrations of DTX [18]. While high micromolar DTX concentrations are detected in plasma of cancer patients early after drug administration, typically they drop to the low nanomolar range a few days post-treatment, which may prevent induction of robust and sustained tumor cell apoptosis [66]. Consistent with this, a recent study indicated that intratumoral accumulation of DTX and CBZ is key for their efficacy, with development of DTX resistance occurring if accumulation of this drug is insufficient [67].

The limited caspase-3 processing and LEDGF/p75 cleavage in taxane treated mCRPC cells observed in this study is consistent with the recent observation that DTX is
a poor inducer of caspase-dependent apoptosis in DU145 cells [68], and our previous observation that DTX induces both caspase-dependent and caspase-independent lysosomal cell death in PC3 cells [18]. It is likely that induction of both caspase-dependent and independent cell death by taxanes occurs in parallel in the tumor microenvironment, and that depending on the cellular context and local drug concentration, one cell death mode may be preferred over the other [18, 69, 70]. This then raises the intriguing question of whether there are intrinsic mechanisms in mCRPC tumors, such as upregulation of LEDGF/p75 and other stress oncoproteins, that favor promoting resistance to taxane-induced caspase-independent cell death. Although the mechanisms of cellular resistance to taxanes are not completely understood, current evidence points to tubulin mutations, multidrug transporters, androgen receptor-mutations, and overexpression of transcription factors and stress proteins such as Stat1, Stat3, NF-kB, Hsp27, and Clusterin [49, 50, 53, 54, 71]. It remains to be established, however, whether some of these mechanisms effectively antagonize taxane-induced caspase-independent cell death. If this turns out to be the case, then targeting multiple molecular pathways that protect tumor cells against both caspase-dependent and caspase-independent cell death could be an effective strategy to overcome taxane resistance in mCRPC. Since LEDGF/p75 appears to be a novel promoter of mCRPC cell resistance to taxane-induced caspase-independent cell death, this protein could be considered as a promising therapeutic target to overcome this resistance in combinatorial therapies.

Finally, understanding the complex mechanisms underlying LEDGF/p75-promoted taxane resistance will require a close examination of the regulatory mechanisms controlling the expression of this stress protein during PCa transition to
chemoresistance. To date, known mechanisms of LEDGF/p75 regulation include its activation by transcription factor Sp1 [72, 73], as well as repression by sumoylation, TGF-β, Bcl-2, ERK, and its small splice variant LEDGF/p52 [52, 74–76]. A crosstalk between LEDGF/p75 activation and the STAT3/IL6 inflammatory pathway, implicated in PCa, has also been identified in HaCaT skin cancer cells and in breast cancer cells [41, 77]. Future studies will explore whether acquisition of taxane resistance in mCRPC involves activation or suppression of LEDGF/p75 regulatory mechanisms.
Figure 13. Model illustrating the potential role of LEDGF/p75 in the attenuation of drug-induced caspase-dependent and –independent cell death.
Materials and Methods

Cell Lines, Antibodies and Reagents

The metastatic PCa cell lines DU145 (brain metastasis) and PC3 (bone metastasis) were purchased from the American Type Culture Collection (ATCC) (Cat.# HTB-81 and Cat.# CRL-1435, respectively). Cells were cultured following supplier’s instructions in RPMI medium (Life Technologies – Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and gentamicin. Cells were maintained in a humidified incubator with 5% CO2 at 37°C. The DTX-resistant cell line variants were developed as described previously [20]. Briefly, DU145 and PC3 cells were cultured in media containing 1 nM DTX and then surviving cells were passaged four times before increasing the concentration of DTX. This was repeated until resistant cells could be maintained with minimal cell death in media containing 10 nM DTX.

The following commercially acquired antibodies were used: rabbit polyclonal anti-LEDGF/p75 (1:1000, Bethyl Laboratories Inc. catalog# A300-848A); rabbit monoclonal anti-β-actin (1:5000, Cell Signaling catalog # 5125); rabbit polyclonal anti-cleaved caspase-3 (1:1000, Cell Signaling catalog # 9661); rabbit monoclonal anti- P-gp/MDR1/ABCB1 (1:1000, Cell Signaling catalog # 13342) and horseradish peroxidase (HRP)-labeled secondary IgG antibodies (goat anti-rabbit IgG, 1:5000, Thermo Fisher Scientific catalog # 31466; goat anti- human IgG/IgA/IgM, 1:5000, Thermo Fisher Scientific catalog # A18847). The human autoantibody to LEDGF/ p75 (1:200) was from the serum bank of the Casiano Laboratory at the Center for Health Disparities and Molecular Medicine at Loma Linda University School of Medicine. The following
cytotoxic drugs were used: DTX (LC-Laboratories), PTX (Sigma-Aldrich), and CBZ (Sanofi-Aventis). TNF-related apoptosis inducing ligand (TRAIL) was purchased from Peprotech and Actinomycin D was purchased from R&D Systems.

**Viability Assays**

PCa cells were treated with the different taxane drugs at the selected concentrations for up to 72 hr, or with TRAIL plus 100 ng/ml Actinomycin D for up to 24 hr. Cell morphology was visualized on an Olympus IX70 microscope equipped with Hoffmann Modulation Contrast (Olympus American) and a digital Spot Imaging System (Diagnostic Instruments). To assess viability, cells were seeded in 96-well plates at a density of 1x10^4 cells per well and then treated with each individual drug in at least triplicates. A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) was performed as described previously [18]. Absorbance was measured at 450 nm using a µQuant microplate reader (Bio-tek Instruments). Values were normalized to the absorbance obtained for the untreated, control cells. The approximate drug half-maximal effective concentration (EC50) was determined using the plotted values of the dose response curve. Each value represents the mean value of at least three different experiments in triplicates. The standard error of the mean (SEM) was calculated for each value.

**Quantitative Real-Time PCR**

Quantitative Real-Time PCR (qPCR) was performed as described previously [28]. Briefly, Total RNA was extracted from cells using the RNeasy plus mini kit (Qiagen).
The iScript cDNA synthesis kit (BioRad) was used to reverse transcribe RNA (0.5 µg) into cDNA. qPCR was performed using the MyiQ real-time PCR detection system with primers using iQ SYBR Green Supermix (BioRad) following manufacturers’ recommendations. Primer sequences for LEDGF/p75 were designed using the Primer3 software. Forward sequence (5’ to 3’) was TGCTTTTCCAGACATGGTTGT and reverse sequence (3’ to 5’) was CCCACAAACAGTGAAAAGACAG. Primers were commercially synthetized by Integrated DNA Technologies (IDT). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used for normalization. Data was normalized to values of corresponding controls and analyzed in three different experiments, each in triplicates.

**Immunoblotting Procedures**

Immunoblotting was performed as described previously [28]. Briefly, equal amounts of protein from whole cell lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE 4–12%, Thermo Fisher Scientific) and transferred into polyvinyl difluoride (PVDF) membranes (Millipore). Membranes were blocked in 5% dry milk or 5% Bovine Serum Albumin (BSA), depending on the primary antibody, prepared in TBS-T buffer (20 mM Tris- HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). Membranes were then probed individually with primary antibodies and corresponding secondary antibodies and washed several times with TBS-T between each antibody application. Enhanced chemiluminescence (ECL) was used to detect immunoreactive protein bands. For this, the ECL Western Blotting Substrate (Thermo Fisher Scientific Pierce, catalog # 32106) was added to the antibody-protein
surface of each membrane, followed by incubation for 5 minutes. Membranes were then transferred to autoradiography cassettes and exposed to autoradiography films for different lengths of time to ensure accurate detection of immunoreactive protein bands.

**Indirect Immunofluorescence Microscopy**

To visualize endogenous LEDGF/p75 expression, cells were grown on coverslips, washed with PBS, then fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100. Cells were then incubated with human anti-LEDGF/p75 serum autoantibody [47] at 1:200 dilution for one hour in a humid chamber. After washes with PBS, cells were incubated with a FITC-conjugated goat anti-human IgG (H+L) secondary antibody (1:200, Thermo Fisher Scientific catalog # 62-7111), used at 1:200 dilution. Cells were mounted and counterstained with medium containing 4’,6-diamidino-2- phenylindole (DAPI) (Vectashield). Images were acquired in a BioRevo Keyence BZ-9000 fluorescent microscope (Keyence). All images corresponding to a particular fluorescent dye were obtained using the same parameters.

**RNA Interference-Mediated Knockdown of LEDGF/p75 in PCa Cells**

To achieve transient knockdown of LEDGF/p75 in our cellular models, specific short inhibitory RNAs (siRNAs) were used as described previously [20, 33]. Specifically, the si-LEDGF/p75 sequence corresponded to nucleotide sequence 1340–1360 (‘5-AGACAGC AUGAGAAGCGAdTdT-3’), present in a region in the C-terminus of LEDGF/p75 that is not shared by its short alternative splice variant LEDGF/p52. Cells were transfected with 100 nM siRNAs using Oligofectamine (Life Technologies)
following manufacturer’s instruction. A scrambled siRNA duplex (siSD, Dharmacon) was used as a negative control.

**Clonogenic Assays**

Cells with and without LEDGF/p75 knockdown were cultured at a density of 1x10^3 per well in 6-well plates and treated with increasing concentrations of DTX or CBZ, or a single concentration of TRAIL combined with Actinomycin D. Cells were grown for 12 days, which is when the surviving colonies were visible. After removing the media, the colonies were washed with PBS and fixed using a 3:1 methanol:acetic acid solution. PBS was used to wash the remaining fixing solution and then a solution containing 0.5% crystal violet diluted in methanol was then added to stain the colonies. Finally, the crystal violet was removed by washing with water and plates were air dried overnight. Images of each individual plate were acquired using the VisionWorks Acquisition and Analysis software in a GelDoc-It^2 imager (UVP, Analytik Jena Company). The parameters to obtain the images were the same for all the plates. The Automated Colony Counting capability of the software was used to count each colony in the individual wells using the same parameters for each plate. At least three plates from three independent experiments were used for quantification of colonies under a particular experimental condition or treatment.

**Statistical Analysis**

The Student’s t-test (paired, two-tailed) was used to evaluate differences between treatment and control groups using Microsoft Excel. Differences were considered statistically significant at P values equal or below 0.05.
References


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CHAPTER THREE
SELECTED UNPUBLISHED DATA

Possible Role of LEDGF/p75 in Protecting PCa Cells Against Necroptosis

Understanding of the molecular mechanisms involved in resistance to both apoptotic and non-apoptotic cell death pathways is essential for the development of innovative therapies and to maximize their clinical potential [1]. Necroptotic (programmed necrosis) and necrotic (accidental necrosis) cell death, share the same morphological features such as lysosomal membrane permeabilization (LMP) and plasma membrane permeabilization, and both can be induced by severe oxidative stress and operate in tumor cells [2]. In a normal context, the release of pro-inflammatory signals from necrotic/necroptotic cells into the extracellular microenvironment initiates a pro-inflammatory response that might not be beneficial to healthy tissue [3]. However, in cancerous tissue a pro-inflammatory response to necroptotic/necrotic cells could further promote tumor development because inflammatory cells are capable of inducing signals leading to increase in angiogenesis, cell proliferation, and invasiveness [3]. For example, IL6, a cytokine that is released by many types of pro-inflammatory cells, including necrotic cells, has been implicated in PCa chemoresistance [4].

Work from our laboratory, including data shown in this dissertation, has provided evidence that LEDGF/p75 upregulation protect cancer cells against inducers of caspase-independent cell death, and that this protein needs to stay structurally intact to exert its protective effects [5-9]. On the other hand, cells treated with the classical apoptosis inducers TRAIL (TNF-related apoptosis inducing ligand) or STS (staurosporine), which activate caspase-dependent apoptosis, die regardless of the level of expression of this
protein [6-7, 9]. The reason is that during apoptosis caspase-3 cleaves LEDGF/p75 into a fragment of 65 kD that lacks the pro-survival activity of the intact protein and enhances cell death under stress conditions [5-8]. Also, p52, the short alternative splice variant of LEDGF/p75 is cleaved by caspase-3 during apoptosis into a p38 fragment that abrogates the survival functions of LEDGF/p75 by abolishing its transcriptional activity [8].

In Chapter 2, Figure 11, we clearly observed cleavage of LEDGF/p75 when PCa cells were treated with TRAIL, but minimal or no cleavage when the cells were treated with the taxane drugs DTX and CBZ [7]. Consistent with this, we did not observe elevated levels of apoptotic cells or processing of caspase-3 into its large and small active subunits in cells treated with these taxanes (Chapter 2, Figure 11). However, caspase-3 activation and cellular apoptotic morphology were clearly observed when PCa cells were treated with TRAIL. This was interpreted as that in our specific PCa cellular models the taxanes are activating a caspase-independent mode of cell death, thus explaining the minimal appearance of apoptotic cleavage fragments of LEDGF/p75 and the fact that this protein stays largely intact during taxane treatment. This could also explain in part why the cells are protected when treated with the taxanes, since by remaining structurally intact LEDGF/p75 is functionally active.

These findings are supported by previous observations from our group in which DTX treatment induced caspase-independent cell death characterized by LMP and cathepsin release in PCa cells treated with DTX, and that enforced overexpression of this protein in these cells protected them against DTX [9]. We also showed recently that LEDGF/p75 overexpression in PCa cells treated with tert-butyl hydrogen peroxide
(TBHP), a stable homologue of hydrogen peroxide widely utilized to induce oxidative stress induced-necrosis, was able to attenuate cell death [6].

If LEDGF/p75 is acting a “guardian of the lysosome” as suggested previously [9-10], this protein could also be involved in protecting cells against physiological pro-inflammatory modes of caspase-independent cell death such as necroptosis [2-3]. To test this hypothesis, we designed a series of experiments in which we treated the PCa cell lines PC3, PC3-DR, DU145, and DU145-DR with the apoptotic inducer TRAIL in the presence or absence of the broad caspase-inhibitor zVAD-fmk. By inactivating TRAIL-induced caspase-dependent apoptosis with this inhibitor, we primed the cells to activate the necroptotic cell death pathway as described previously [11-12]. We pre-treated the PCa cells for 2 hours with 20μM zVAD and then treated them with 100ng/ml TRAIL in combination with 20μM zVAD. We measured cell viability at 72 hrs and 96 hrs after addition of z-VAD to determine if cells could recover from TRAIL-induced apoptosis.

Our results showed that PC3 cells (low LEDGF/p75 expression) and DTX-resistant PC3-DR cells (high LEDGF/p75 expression) treated with TRAIL for 72 hrs (Figure 14A) and 96 hrs (Figure 14B), showed low survival regardless of LEDGF/p75 expression levels. However, when we pre-treated cells with the TRAIL/z-VAD combination, we observed a slight but significant recovery in cell viability at both 72 hrs and 96 hrs, especially in the PC3-DR cells, which have high endogenous levels of LEDGF/p75. This could be more clearly observed at 96 hrs, in which there was a significant recovery in viability in the PC3-DR cells treated with TRAIL/zVAD compared to the PC3 cells with the same treatment conditions (Figure 14B).
Figure 14. Assessment of cell viability as measured by MTT assay in PC3 and PC3-DR cells treated with TRAIL or TRAIL with zVAD. PC3 (red bars) and PC3-DR cells (blue bars) treated with 100ng/ml TRAIL or 100ng/ml TRAIL with 20uM of zVAD for 72 hrs (A) and 96 hrs (B). Statistical significance was determined by comparing the values between the groups treated with TRAIL or TRAIL + zVAD and between PC3 and PC3-DR cells treated with TRAIL + zVAD respectively, using Student’s t-test (*p<0.05).
We performed experiments under exactly the same conditions in DU145 and DU145DR cells and measured cell viability at 72 hrs (Figure 15A) and 96 hrs (Figure 15B). Similar to what we observed previously the PC3 and PC3-DR cells, a significant recovery in cell viability was observed when DU145 and DU145-DR cells were treated with the TRAIL/z-VAD combination, compared to cells treated only with TRAIL. This significant recovery in cell viability was observed at both 72 hrs (Figure 15A) and 96 hrs (Figure 15B). However, the recovery was more evident when cells were treated with the TRAIL/z-VAD combination at 96 hrs in the DU145-DR cells, which have higher endogenous levels of the LEDGF/p75 protein, when compared with DU145 cells treated under the same conditions although it was not statistically significant (Figure 15B).

While we cannot determine from the present data that the protection of the DR cells against TRAIL/z-VAD induced necroptosis was due to elevated expression of LEDGF/p75 in these cells, we speculate that upregulation of this protein may contribute to protection of PCa cells under conditions in which there is activation of necroptotic-cell death. We induced necroptosis with TRAIL/z-VAD, a combination that has been demonstrated to induce necroptosis in cancer cells. We can infer based on these and published data that under drug treatments that fail to effectively activate caspases, thus leaving LEDGF/p75 uncleaved by caspases, this protein will remain intact and able to exert its cellular protective functions, including chemoresistance.
Figure 15. Assessment of cell viability as measured by MTT assay in DU145 and DU145-DR cells treated with TRAIL or TRAIL with zVAD. DU145 (green bars) and DU145-DR cells (orange bars) treated with 100ng/ml TRAIL or 100ng/ml TRAIL with 20uM of zVAD for 72 hrs (A) and 96 hrs (B). Statistical significance was determined by comparing the values between the groups treated with TRAIL or TRAIL + zVAD and between DU145 and DU145-DR cells treated with TRAIL + zVAD respectively, using Student’s t-test (*p<0.05).
LEDGF/p75 Overexpression Leads to Resistance to Doxorubicin Treatment

In the previous chapter, we showed that the DTX resistant PC3-DR and DU145-DR cells were not only resistant to this drug, but also to the other clinically relevant taxanes PTX and CBZ when compared to the drug-sensitive PC3 and DU145, respectively. When we treated the cells with the classical apoptotic inducer TRAIL they were sensitive, as evidenced by the robust apoptosis detected in all cell lines. These results suggested that the protection conferred in part by LEDGF/p75 overexpression is drug-specific. TRAIL sensitivity was explained by previous studies showing that caspase-3 cleaves LEDGF/p75 into a fragment of 65 kD [5]. This fragments does not possess the pro-survival activities of the intact protein and even exacerbates cell death under stress conditions [5]. We also studied the response of the drug-resistant and – sensitive cells to another drug, doxorubicin (DOXO). This drug is a member of the anthracyclines, a class of drugs capable of intercalating DNA and directly affecting transcription and replication [13]. DOXO is not utilized in typical chemotherapeutic regimens due to its toxicity. However, there are reports of the utilization of this drug for the treatment of CRPC, although severe toxicity has been reported when used alongside an aggressive treatment regime [13]. To determine if our taxane resistant PCa cells were also sensitive to DOXO, we treated PC3, PC3-DR, DU145 and DU145-DR with increasing concentrations of DOXO and measured cell viability 72 hr after the initial exposure. PC3-DR cells exhibited significantly increased cell viability when compared to the PC3 cells at the 500 nM and 1000 nM range (Figure 16A). DU145-DR cells also exhibited increased viability when treated with DOXO, compared to the drug sensitive
DU145 cells (Figure 16B). However, the data for DU145 cells is the average of two experiments in triplicates. Additional experiments in this specific range of concentrations are necessary with the DU145 and DU145-DR cells to reach statistical significance.

The data presented in Figure 16 indicate that the DTX-resistant cells, which have high LEDGF/p75 expression, are more resistant to DOXO compared to sensitive cells. These observations coincide with data by other groups showing that overexpression of LEDGF/p75 in MCF-7 cells protected these cells against LMP-inducing agents such as siramesine, etoposide, and DOXO [10]. This is also consistent with the observation that osteosarcoma cells depleted of LEDGF/p75 were incapable of effectively repairing DNA damage induced by DOXO, implicating this protein in DNA damage repair [14].

Because of the significance of taxanes in the clinical treatment of PCa, and the minimal clinical relevance of DOXO in the treatment of this malignancy, we opted not to continue performing experiments using DOXO, and to focus our efforts on studying the effects of LEDGF/p75 targeting on the sensitization of DTX-resistant PCa cells to taxanes.
Figure 16. PC3-DR (A) and DU145-DR (B) cells are resistant to DOXO. Assessment of cell viability as measured by MTT assay in PC3 (red triangles), PC3-DR (crosses), DU145 (orange diamonds), and DU145-DR (green squares) cells treated with increasing concentrations of DOXO (5nM, 10nM, 50nM, 500nM, and 1000nM) for up to 72 hr. Graph A (PC3) represents the average of at least three different experiments in triplicates normalized to untreated controls. Graph B (DU145) represents the average of two different experiments in triplicates normalized to untreated controls. Standard error of the mean (SEM) was calculated in both graphs. Utilized Student t-test (*p<0.05).
Initial Repurposing of HIV-Based Inhibitors to Target LEDGF/p75 in PCa

The development of cytotoxic drugs and combinatorial regimens have resulted in an improved quality of life and prognosis of cancer patients [15]. However, there is still a need to develop innovative and targeted therapeutic approaches to circumvent chemotherapy resistance [16] As mentioned previously, LEDGF/p75 is a well-established cellular co-factor for HIV-1 replication and is currently being extensively studied as a druggable target. The LEDGF/p75–HIV IN interaction or the interaction of LEDGF/p75 with other oncoproteins in the context of cancer are ideal therapeutic targets since they provide new opportunities for the development of small molecule inhibitor (SMI)-based treatments [16].

Recently, SMIs have been developed to disrupt LEDGF/p75-HIV-IN interaction resulting in decrease viral replication [17-18]. Its role in HIV infection and relevance in cancer makes LEDGF/p75 an attractive therapeutic target in both HIV-AIDS and cancer. Inhibitors designed to disrupt LEDGF/p75 interactions with HIV-IN through its IBD can also be repurposed or repositioned for cancer treatment because in cancer cells LEDGF/p75 interacts with other cancer-related proteins precisely through the IBD. For example, small peptides were demonstrated to effectively disrupt the interaction between LEDGF/p75 and the MLL/menin complex in leukemia cells, decreasing MLL-fusion-mediated transformation [19].

Our collaborator, Dr. Nouri Neamati (University of Michigan), and his team described the design and discovery of novel SMIs that inhibited the interaction between LEDGF/p75 and HIV-IN (Figure 17) [20].
Figure 17. Disruption of the protein-protein interaction between LEDGF/p75 and HIV-IN by SMIs (Adapted from Suzuki et al. 2011, Sanchez et al, 2012) [20].
Using LEDGF/p75 IBD-based pharmacophore models, these researchers identified acylhydrazones that effectively disrupted this interaction. This class of compounds are used to treat tuberculosis and have also been demonstrated to possess antiretroviral activity [20]. We started initial studies aimed at evaluating the potential of repurposing these SMIs to target LEDGF/p75 in order to sensitize CRPC cells to chemotherapy.

In collaboration with Dr. Neamati, we initially evaluated the cytotoxicity of approximately 130 of these acylhydrazones compounds in PC3, PC3-DR without the presence of any other drugs in order to identify which ones showed activity or decreased cell viability (Figure 18 A-G). We treated the cells with the compounds at 1μM, 5μM and 10μM and measured cell viability using MTT at 72 hr. For example, compounds 13 and 14 in Figure 18A, showed robust cytotoxicity in the PC3 cells but showed minimal toxicity in the PC3-DR cells. This could be explained by the high endogenous levels of LEDGF/p75 in the PC3-DR cells versus in the lower expression in the PC3 cells and the ratio of protein/compound. Perhaps a higher concentration of these compounds might be toxic by itself, but since at 10μM they showed activity in the PC3 cells, this was an indicator that these compound might be active when combined with DTX. From this initially tested group, we then selected several compounds that showed activity and evaluated them in PC3-DR cells in combination with 10 nM of DTX for 72 hrs.

Our goal was to identify candidate compounds that either showed cytotoxicity alone in both sensitive and resistant CRPC cells or that did not show cytotoxicity alone but did exhibit cytotoxicity in the chemoresistant cells the presence of 10 nM DTX,
which is the maintenance concentration of the drug-resistant cells. Figure 19 shows examples of the type of studies we want to further pursue. In these experiments we treated PC3-DR cells with 10μM of each compound in combination with 10nM DTX, and after 72 hrs we assessed cell viability utilizing MTT assay. In these preliminary results, SMIs 13, 14 and 80 showed no cytotoxicity in PC3-DR cells when used alone but their effects were more dramatic in combination with DTX. Additional experiments to determine the IC50 of these compounds in the presence and absence of DTX, and achieve statistical significance, will be performed in studies beyond the scope of this dissertation. These experiments will also be expanded to additional PCa cell lines with variable levels of LEDGF/p75 such as DU145 and DU145-DR cells, and cell lines with transient and stable knockdown of LEDGF/p75.

To further select the lead SMIs, we compiled data from Sanchez et al. [20], in which some of these compounds also showed anticancer activity as determined by their IC50 and/or cytotoxicity in HCT116 (colorectal carcinoma) cells (Table 1). These will be the first to be tested but others will be selected considering the previous preliminary experiments. Once our group identifies lead SMIs that exert cytotoxicity in cells overexpressing LEDGF/p75, such as the drug-resistant PC3-DR and DU145-DR, in the presence of taxanes, then in vitro binding studies will be performed to determine the specific binding of these lead SMIs to LEDGF/p75.
Figure 18. Initial screening of SMIs in PC3 and PC3-DR cells. Cells were treated with 10uM of SMIs to identify candidate compounds that show activity. Viability was assessed 72hrs after treatment utilizing MTT.
Figure 19. Co-treatment with selected SMI’s and 10nM of DTX. PC3-DR cells were treated with 10uM of each compound in combination with 10nM of DTX, their maintenance drug concentration. Viability was assessed 72hrs after treatment utilizing MTT. Average of three individual experiments in triplicates normalized to untreated controls. Standard error of the mean (SEM) was calculated. Utilized student t-test (*p<0.05).
Table 1. Selected compounds for further studies. Additional data obtained from Sanchez et al, 2013 [20].

<table>
<thead>
<tr>
<th>Compound Identifier and structure</th>
<th>LEDGF/p75-IN IC50 uM</th>
<th>HCT116 +/- p53 IC50 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 - 31B12 (84) En-T5755296</td>
<td>4 ± 2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>91 - EN10K 31C12 En-T5755298</td>
<td>2 ± 1</td>
<td>6</td>
</tr>
<tr>
<td>14 - EN10K 31A13 (89) En-T5756746</td>
<td>7 ± 3</td>
<td>1.5</td>
</tr>
<tr>
<td>82 - EN10K 31A12 En-T5755295</td>
<td>&gt;50</td>
<td>&lt;1</td>
</tr>
<tr>
<td>B31E11 (110) T5755287</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>60b1 (118)</td>
<td>23 ± 3</td>
<td></td>
</tr>
</tbody>
</table>
Our ultimate goal in these follow-up studies is to identify and characterize a panel of lead SMIs that abrogate LEDGF/p75 pro-survival functions in chemoresistant cells in the presence of DTX. We hypothesize that these compounds disrupt LEDGF/p75 survival functions by binding to its IBD and hindering its interaction with other co-transcription factors. This would result in decreased LEDGF/p75-mediated transactivation of survival genes. Studies focused on the functional capabilities of this protein will be performed to investigate if the transactivation of target genes, such as Hsp27, is affected. Follow-up studies could also explore if the interaction of LEDGF/p75 with other transcription factors or co-activators, such as Menin and MeCP2, is disrupted. Finally, by studying the structure and functional groups of the lead SMIs, in the context of their cytotoxic functions, we will be able to identify if certain chemical moieties in these compounds are more relevant and show increased activity.
References


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CHAPTER FOUR
OVERALL DISCUSSION

Prostate cancer (PCa) is the most commonly diagnosed cancer in U.S. males and the third leading cause of cancer deaths in this population [1]. CRPC, the advanced form of this disease, is characterized by biochemical and radiographic progression despite hormonal manipulation, and is associated with poor prognosis [2-3]. Progress in understandings the mechanisms leading to CRPC have resulted in the development of new therapeutic strategies and regimens, including the anti-androgen drugs abiraterone acetate and enzalutamide, the immunotherapeutic vaccine sipuleucel-T, the immunotherapeutic antibody denosumab to prevent bone fractures, and the chemotherapeutic taxane drugs DTX and CBZ [4]. Despite the different FDA-approved therapeutic options, from only DTX in 2004 to six different therapies available in 2016, managing CRPC is still challenging due mainly to the limited clinical and survival benefits of these treatments caused by primary and acquired resistance [3]. There is an urgent unmet need in understanding what would be the most beneficial treatment options and combinatorial therapies, and the most appropriate sequence of the therapy administration [5]. Another layer of complexity is the heterogeneity in the presentation of PCa, from undiagnosed indolent to rapidly spreading and lethal tumors, and the broad spectrum in its biological and clinical behavior, even within the same patient [6]. Efforts to understand the genomic landscape of PCa and the genomic diversity between localized and aggressive metastatic disease, along with the identification of potentially druggable aberrations, are still needed to identify potential therapeutic targets [6].
In this dissertation research we sought to further establish the role of LEDGF/p75 in PCa taxane resistance and its potential as a novel therapeutic target for overcoming chemoresistance. As reviewed in the Introduction, research from our group, including the published data presented in Chapter 2 (hereafter cited as ref. 18), and others supports the role of this emerging stress oncoprotein protein in promoting tumorigenesis and tumor aggressive properties, including chemoresistance, in various human cancer types [7-17]. While further studies are necessary to completely understand the mechanisms by which LEDGF/p75 protects cancer cells against environmental stressors, including chemotherapeutic drugs, we have demonstrated that LEDGF/p75 overexpression protects cells against caspase-independent cell death caused by strong inducers of oxidative stress and LMP such as chemotherapeutic drugs and oxidants [9, 11].

We provided evidence in Chapter 2 that the clinically relevant taxane drugs DTX and CBZ can activate a caspase-independent mode of cell death, as demonstrated by minimal caspase-3 processing, LEDGF/p75 cleavage, and apoptotic morphology in PCa cells exposed to these drugs. Furthermore, our unpublished results in which we treated our PCa cells with TRAIL in the presence of the broad caspase-inhibitor zVAD-fmk, indicate that LEDGF/p75 overexpression might confer resistance to therapeutic drugs under conditions in which caspases are inactivated. These observations are relevant due to increasing evidence of the unfolding role of caspase-independent modes of cell death, such as necrotic/necroptotic pathways, in the development of drug resistance depending on the tumor microenviroment [18]. Efforts during the past decade utilizing single pathway anti-apoptotic agents in order to elicit an apoptotic response in cancer cells have not been successful. Thus, feasible and successful treatment strategies have to consider
the crosstalk between multiple survival networks including survival proteins and other modes of cell death [19].

Previous work from our group demonstrated that LEDGF/p75 overexpression attenuated DTX-induced caspase-independent cell death associated with LMP in PC3 cells [9], but does not protect against caspase-dependent cell death induced by TRAIL or STS [9,11]. Our observation of minimal caspase activation and/or activation of a caspase-independent pathway by DTX is supported by recent observations from another group showing that DTX induced minimal caspase activation in DU145 cells [20]. In light of this evidence, we have to consider the possibility that cancer cells treated with DTX and CBZ may activate both caspase-dependent and –independent cell death mechanisms of in an effort to effectively respond to cytotoxic insults [21-22]. The development of resistance to non therapy-specific and therapy-specific insults, including activating genes to antagonize a specific mode of cell death, highlights the plasticity of cancer cells to adapt to the environmental conditions [3].

Although DTX is still the preferred cytotoxic agent utilized for the treatment of CRPC, yielding survival benefits, around half of the patients do not respond to this therapy, and those who initially respond eventually develop resistance, ultimately succumbing to the disease [23]. Resistance to taxanes is not completely understood, but evidence points out to tubulin mutations, multidrug transporters, androgen receptor-mutations, and overexpression of transcription factors and stress proteins such as Stat1, Stat3, NF-kB, Hsp27, and Clusterin [23-28]. Also, tumor growth and chemoresistance are aided by the disablement of cell death responses and the interplay between microenvironmental or intracellular factors that antagonize or promote the different
modes of cell deaths, particularly apoptosis, necrosis and autophagy [29]. Whether the observed resistance is driven by extrinsic or intrinsic factors, such as the upregulation of LEDGF/p75 and other stress oncoproteins, evidence supports the concept that PCa progression towards an aggressive state characterized by complete therapy resistance is associated with increased molecular aberrations that gives the tumor the advantage to circumvent the anti-tumor effects of therapy [6]. Until we fully comprehend how all these molecular alterations affect therapeutic outcome, logically the best approach would be to simultaneously or sequentially target multiple molecular pathways that maintain resistance to both caspase-dependent and –independent cell death.

For the present studies, we used CRPC cellular models (PC3 and DU145) that were treated with increasing concentrations of DTX and selected over time for their acquired resistance to this drug. These cells naturally overexpress LEDGF/p75 endogenously as they acquire this resistance, suggesting that this stress protein may contribute to promoting the selection of surviving cells in the presence of DTX. Although other mechanisms of resistance and other survival proteins are also likely overexpressed in these cells, we were interested in the role of LEDGF/p75 due to previous observations from our group linking its enforced overexpression (plasmid-mediated) to DTX resistance in PCa cells [9]. We observed that as these cells acquired resistance to DTX, they also became resistance to the other clinical taxanes, PTX and CBZ, as well as the DNA-intercalating drug DOXO (Chapter 3, Figure 3). This observation is consistent with the clinical observation that patients who develop resistance to DTX therapy eventually also develop resistance to other drugs, including CBZ [3-5]. Resistance to DOXO and other DNA damaging drugs in cells
overexpressing LEDGF/p75 has been previously documented, given the function of this protein in DNA damage repair [8,10,14]. As shown in Chapter 2, both DTX-sensitive and -resistant cell lines exhibited robust sensitivity to the apoptosis inducing death ligand TRAIL. This suggests that the cellular protective functions in these cellular models of DTX resistance could be drug-specific. The exquisite sensitivity to apoptosis induced by death ligands such as TRAIL in chemoresistant PCa cells could be exploited as a strategy to bypass resistance, however the high toxicity of these ligands tempers their clinical utility as discussed below.

LEDGF/p75 appears to be involved in cellular resistance to caspase-independent cell death but not to caspase-dependent apoptosis. This is supported by observations from our group and others that overexpression of this protein protects cells against taxane-induced, caspase-independent cell death associated with LMP, and also against oxidative-stress induced necrosis induced by TBHP [9, 11]. As we demonstrated in our studies, PCa cells overexpressing LEDGF/p75 did not appear to be resistant to the classical apoptotic inducers TRAIL or STS regardless of the levels of this protein [9, 10, 11]. These observations are concurrent with other studies from our laboratory that showed that during caspase-dependent cell death, caspase-3 cleaves LEDGF/p75 into a fragment of 65kD that lacks the pro-survival activity of the intact fragment and enhances cellular death under conditions of stress [7, 11]. Also, when the short alternative splice variant of LEDGF/p75, p52, was cleaved by caspase-3, it abrogated the survival functions of LEDGF/p75 by abolishing its transcriptional activity [30]. Thus, we can reach the conclusion that LEDGF/p75 is not a classical anti-apoptotic protein such as Survivin, Bcl-2, and Clusterin, which directly inhibit intracellular processes associated
with apoptosis, but rather an inhibitor of stress-induced cell death involving LMP such as necrotic cell death. However, it is plausible that LEDGF/p75 could promote protection against apoptosis if this mode of cell death occurs downstream of events antagonized by this protein such as DNA damage and LMP [9, 10, 14].

In agreement with this conclusion, we also showed that in conditions in which the cells are primed to undergo necroptosis [31-33], drug-resistant PCa cells, which have high endogenous levels of LEDGF/p75, are more resistant to TRAIL-induced cell death in the absence of caspases than the drug sensitive cells. These observations further implicate LEDGF/p75 in resistance against caspase-independent cell death, possibly necroptosis, although this possibility needs to be further explored. Necroptosis is a recently described regulated form of programmed necrosis mediated by receptor interacting protein kinases 1 and 3 (RIPK1 and RIPK3) and its substrate mixed lineage kinase like (MLKL) [31]. Phosphorylation and trimerization of RIPK1,3 in the absence of caspase activation leads to phosphorylation of MLKL, which in turn oligomerizes in the plasma membrane, leading to its permeabilization, LMP, and cytoplasmic destruction [34]. Evidence supports that necroptosis could have a pro-inflammatory function by acting as an initiation signal and contributing to the amplification of signaling leading to production of cytokines [35]. By inhibiting necroptosis, perhaps by protecting oxidative damage to lysosomal membranes, LEDGF/p75 might be able to reduce this inflammatory response and subsequent tissue damage. However, in order to unambiguously demonstrate a role for LEDGF/p75 in PCa cell resistance to drug-induced necroptosis, additional studies will be necessary utilizing specific necroptosis inhibitors such as necrostatin (Nec-1, a RIPK1 inhibitor), GSKs (RIPK3 inhibitors), and Necrosulfanomide (MLKL...
inhibitor), in cells with and without LEDGF/p75 overexpression and in the presence or absence of combinations of z-VAD with different antitumor drugs such as taxanes, TRAIL, doxorubicin, etc. The high complexity and labor intensive nature of these additional studies merits a systematic and comprehensive analysis that is beyond the scope of this dissertation.

If LEDGF/p75 contributes to selective drug resistance and is inactivated by caspases in PCa and other cancer types, then induction of tumor cell apoptosis via death receptors could be used to bypass the pro-survival activity of LEDGF/p75 and other similar survival proteins. The induction of apoptosis is a therapeutic approach currently being studied since mutation rates in the apoptotic receptors are low, suggesting that the apoptotic machinery in tumor cells is relatively intact [36]. This could be used as an advantage to target these cells. However, it needs to be recognized that in spite of the broad knowledge on mechanisms of apoptosis generated during the past three decades, we are still lacking effective anti-cancer therapies targeting tumor apoptosis. Efforts to target the extrinsic – i.e. death receptor-mediated- apoptotic pathways have focused on agents targeting the death receptors TRAIL-R1 and TRAIL-R2 because these receptors are more prevalent in cancerous cells than in normal cells [36]. However, reports have also found high levels of these receptors in normal cell types such as hepatocytes, brain tissue and keratinocytes [36]. This could attenuate the clinical effectiveness of targeting these receptors. For example, a recent clinical trial in which a TRAIL-R2 agonist (TAS266) was used reported adverse effects due to hepatotoxicity [37]. This raises concern for the safety of these types of drugs targeting receptors that play a critical role in normal tissues due to the possibility of high toxicity to the patient. Therefore, other
therapeutic options targeting molecular pathways that are critical for tumor growth but not necessarily for normal tissue growth and function have to be explored in order to circumvent therapy resistance.

As mentioned previously, in addition to its role in cancer chemoresistance, LEDGF/p75 plays a pivotal role in facilitating HIV-1 integration and replication through its C-terminal integrase binding domain (IBD) [38]. Considering the therapeutic potential of disrupting its protein-protein interactions and the pro-survival functions of LEDGF/p75, which are largely dependent on the structural integrity of the IBD, various studies aimed at targeting this protein have demonstrated that it is a druggable target [38-42]. For instance, studies from other laboratories have demonstrated that targeting LEDGF/p75 decreased tumorigenic potential in DU145 cells [13, 14], and sensitized cancer cells (non-PCa) to non-taxane drugs [14], implicating LEDGF/p75 as a contributor in the development of chemotherapy resistance. Importantly, LEDGF/p75 does not appear to be essential for normal cellular viability since PSIP1/LEDGF/p75−/− knockout mice were viable despite suffering multiple developmental skeletal malformations that led to an increase in perinatal mortality [43]. In addition, cell lines with stable knockdown of LEDGF/p75 have been effectively developed [44-46] albeit with the caveat that they have been selected from a surviving pool derived from a larger population and have likely developed compensatory mechanisms to survive in the absence of this protein. Taken together, however, these observations validate LEDGF/p75 as a potential therapeutic target in diseases in which this protein play a critical role such as HIV-AIDS and cancer.

If LEDGF/p75 is upregulated during the development of taxane chemoresistance,
the reasoning is that targeting this protein in pre-clinical models of CRPC would sensitize the chemoresistant cells when treated in combination with chemotherapy. Towards this goal, we showed that transient depletion LEDGF/p75 reduced the clonogenic potential of both sensitive and chemoresistant PC3 and DU145 cell lines, results consistent with previous observations [10, 13-14]. We also observed that targeting LEDGF/p75 with siRNA in combination with DTX or CBZ treatment significantly reduced the clonogenicity of both DU145-DR and PC3-DR cells, with LEDGF/p75 knockdown sensitizing the DU145-DR cells more robustly than PC3-DR cells. This would be consistent with previous studies that showed that LEDGF/p75 silencing abrogated aggressive properties in DU145 cells [13]. As previously mentioned, we could not effectively compare the EC50 values of LEDGF/p75 knockdown alone versus knockdown in combination with taxanes due to the limitations in the drug range concentrations selected for these experiments. Moreover, as discussed in Chapter 2, synergism between the effects of LEDGF/p75 knockdown alone and knockdown plus taxanes could not be calculated because such studies would required a broad range of concentrations for both treatments, which cannot be achieved with protein knockdown. However, our results provide “proof of principle” for the concept that targeting LEDGF/p75 in combination with taxanes could be a promising strategy for circumventing chemoresistance in CRPC.

In our studies we also initiated the evaluation of SMIs previously identified by our collaborators [40] to target the interaction between HIV-IN and LEDGF/p75 for their potential to target LEDGF/p75 and produce cytotoxicity and overcoming chemoresistance in PCa cells. In these initial studies we were able to identify candidate
SMIs that exhibited cytotoxicity in PCa cells when used alone or in combination with DTX. We hypothesize that some of these SMIs might be binding to the IBD-domain of LEDGF/p75, causing hindrance and possibly disrupting protein-protein interactions with other co-transcription factors that are essential for the transcriptional and survival functions of this protein. We anticipate that these initial studies will be expanded to identify a few candidate LEDGF/p75 inhibitory compounds that show promise for circumventing taxane resistance in pre-clinical models of CRPC.

Although we focused this dissertation work on the role and targeting of LEDGF/p75 in the context of PCa chemoresistance, we also have to consider the interplay between multiple, different survival pathways contributing to chemoresistance since LEDGF/p75 silencing did not completely sensitize resistant PCa cells to DTX and CBZ [Chapter 2]. The identification of these pathways in cellular models and in patient-derived biospecimens will facilitate the identification of patients that will response to therapy, avoiding unnecessary exposure to cytotoxic drugs [21]. We have to consider in our experimental context the contribution of other pro-survival proteins implicated in chemoresistance, including Clusterin, heat shock proteins, multi-drug resistance proteins, inhibitors of apoptosis, etc. [23, 28, 47-49]. Current studies in our laboratory seek to simultaneously target LEDGF/p75 and Clusterin to re-sensitize PC3-DR and DU145-DR cells to DTX.

Another candidate protein for such combinatorial targeting is P-glycoprotein (P-gp), a multi-drug resistance protein and drug transporter that is weakly expressed in normal prostate, but is highly expressed in primary PCa cells and tumor epithelium and has been associated with tumor stage and grade [50]. In our studies we asked the question
of whether LEDGF/p75 depletion affected the expression of P-gp in our taxane-resistant PCa cells. This was prompted by the possibility that LEDGF/p75, acting as a transcription co-activator, could promote the upregulation of P-gp in chemoresistant cells. However, our results indicate that these protein act independently of each other in our chemoresistant cell models since knockdown of LEDGF/p75 did not affect the expression levels of this drug transporter. However, we cannot rule out that P-gp is still regulated by LEDGF/p75 at the transcriptional level but its protein stability is not affected by LEDGF/p75 depletion. Alternatively, we could conclude that PCa cells develop redundancy in their pro-survival and chemoresistance pathways as a strategy to survive against different stressors in specific microenvironmental contexts. For instance, a previous study demonstrated that although P-gp is upregulated in several chemoresistant PCa cells lines, its knockdown only reverses the resistant phenotype in a cell-line dependent manner [51]. Although P-gp is an attractive therapeutic target, targeting of this drug transporter has not been clinically successful due to adverse side effects, toxicity, and altered pharmacokinetics encountered in the studies [51]. The reason for this is that P-gp, like TRAIL receptors, plays important physiological roles in normal cells that when abrogated with inhibitors cause negative side effects in patients [51].

Finally, we also observed marginal caspase-3 processing and LEDGF/p75 cleavage in PCa cells (both sensitive and chemoresistant) treated with DTX and CBZ at relatively high pharmacological plasma concentrations that we documented induced cell death. Previous observations from our laboratory and another group also demonstrated that caspase-3 activity or LEDGF/p75 cleavage could be induced in PC3 and DU145 cells, albeit not very robust, at micromolar concentrations of DTX [9, 13]. Taken
together, these findings suggest that in addition to apoptosis, other mechanisms of cell
death that are activated during taxane treatment could result in the development of
resistance since pro-survival proteins such as LEDGF/p75 may preserve their structural
integrity in non-apoptotic cell death modalities and protect against drug treatment if
overexpressed in the tumors.

Conclusions

The development of redundancy in chemoresistance pathways, likely through
activation of multiple survival proteins and molecular pathways that antagonize various
modes of tumor cell death, presents a serious two-prong challenge in the clinical
treatment of advanced PCa tumors and other tumors: 1) the multiplicity of pathways that
will need to be targeted to circumvent the resistance, and 2) the toxicity that could be
developed by patients after targeting some of these pathways in clinical trials. In spite of
these challenges, and considering the results from the present study and previous studies
from our group and other investigators on LEDGF/p75, we believe that co-targeting of
multiple proteins that are not very critical for the function of normal cells and tissues but
that are overexpressed in chemoresistant tumors and that protect tumor cells against both
caspase-dependent and caspase-independent cell death, is a promising and effective
strategy to overcome taxane resistance in CRPC. We propose that LEDGF/p75 could be
one of these proteins and a novel therapeutic target in chemoresistant CRPC. Future
studies on establishing LEDGF/p75 as a PCa therapeutic target will need to be focused on
establishing the role of this protein in promoting other characteristics of prostate
tumorigenesis such as tumor growth, proliferation, invasiveness, angiogenesis, epithelial
to mesenchymal transition, metastasis to bone, etc. Considering the fact that LEDGF/p75 may not be essential for cell survival under normal conditions, but plays a role in the development and maintenance of chemoresistance, it would be important to establish if this protein is overexpressed as a result of the stressful microenvironmental conditions inside the developing prostate tumor or as a response to the increased endogenous ROS accumulation in tumor cells when patients are treated with chemotherapy. The latest possibility is considerably worrisome because of its implications for the development of highly aggressive disease as a result of chemotherapy. Hence, detailed studies elucidating the functions, targets, and interacting partners of LEDGF/p75 in CRPC are still necessary to fully understand the functions of this stress oncoprotein and the mechanisms by which it promotes PCa chemoresistance. This will be critical in the development of appropriate therapeutic options for chemoresistance PCa involving targeting LEDGF/p75.


CHAPTER FIVE
METHODOLOGY

The contents of this chapter are for the purpose of instructing current and future laboratory members on the methodology utilized in this project. This section will provide key details and insights that are important to reproduce the studies presented in this dissertation and to troubleshoot common problems that could be encountered in follow up studies.

Developing DTX-Resistant Cell Lines

The metastatic PCa cell lines DU145 (brain metastasis) and PC3 (bone metastasis) were purchased from the American Type Culture Collection (ATCC) (Cat.# HTB-81 and Cat.# CRL-1435, respectively). Cells were immediately stored in liquid nitrogen upon arrival until they were ready to be cultured. These were cultured carefully following supplier’s instructions in RPMI medium (Life Technologies – Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and gentamicin. Our media is also supplemented with the addition of the reagent Normocin (Invivogen) to ensure broad antibacterial protection, including mycoplasma. Cells were maintained in a humidified incubator with 5% CO2 at 37°C. It is recommended that after cells have been passaged 4 times, the protein levels of LEDGF/p75 are examined by immunoblotting. Our group has previously demostrated that these have significantly lower levels of LEDGF/p75 than the drug-resistant variants [1]. To ensure the quality and reproducibility of the data derived from future studies, LEDGF/p75 protein levels should be tested after each batch of cells, both sensitive and
resistant, is thawed and re-grown for a few days. This is important because stressed drug sensitive cells may upregulate LEDGF/p75 levels, minimizing the effects of the drug resistant cells in comparison studies.

To ensure quality and viability, cells are collected and frozen when the passage number was low or less than 5 passages. The DTX-resistant cell line variants were developed as described previously by Patterson et al, 2006 [2], but with a few modifications. Briefly, DU145 and PC3 were cultured with the normal medium supplementation but with the addition of 1 nM DTX. The surviving cells were passaged four times before increasing the concentration of DTX. The culture flasks have to be at least 70% re-populated by the surviving cells before they are split and exposed to with increased DTX concentrations. If the cell mortality rate is too high and there are only a few cells left, these can be re-cultured in a small plate or flask. This allows the cells to have important cell-cell contacts that will enable them to grow and multiply. The media should be changed every 48 hours even if the flask is not confluent, to ensure that the cells are properly supplemented with nutrients and DTX. The concentration of DTX was then sequentially increased to 2nM, 4nM, 6nM and finally to 10nM. This is a variation from the original protocol in which the cells were initially treated with 1nM and then with 5.5nM. We found that this drastic increase in the DTX concentration was too harsh on the cells. It was more logical and practical to gradually increase the concentration of DTX in increments of 2nM. This was then repeated until only the cells with the resistant phenotype were selected and could be maintained with minimal cell death in media containing 10 nM DTX. It is important to assess microscopically the morphology of the cells before and after each increment in DTX concentration, because the drug-resistant
cells have a distinct morphology. They are bigger, rounder, and more multinucleated than the parental, drug-sensitive cells. They also have higher messenger and protein levels of stress proteins such as LEDGF/p75, Clusterin, and P-gp. To ensure that the cells maintain this resistant phenotype, they should be grown in the maintenance concentration of 10nM of DTX, and the levels of LEDGF/p75 and Clusterin should be tested after each batch is re-grown.

**Treatment With Different Drugs and Reagents**

In our experiments, PCa cells were treated with the different cytotoxic drugs at increasing concentrations for up to 72 hr, or with 100 ng/ml TRAIL plus Actinomycin D for up to 24 hr. The efficiency of TRAIL in inducing apoptosis is enhanced in the presence of a transcription inhibitor such as Actinomicyn D or a protein synthesis inhibitor such as cycloheximide. To ensure reproducibility and efficiency, drugs should be handled following the instructions given by the manufacturer. For example, to prepare 10nM DTX, dissolve the drug in solid form in the vehicle agent DMSO. It is important to know what solvent to use to dissolve each drug considering their solubility properties. The solubility of DTX in DMSO is 200mg/ml. The molecular weight of DTX is 807.88. To prepare 10ml of a 10mM stock, dissolve 80.788 mg in 10ml of DMSO. Divide in aliquots of 1ml and freeze the aliquots not in use to ensure stability. It is important to dilute each drug in serial dilutions to maintain proportion of drug to media and equal distribution of particulate.

The following cytotoxic drugs were obtained from these companies: DTX (LC-Laboratories, cat# D-1000), PTX (Sigma-Aldrich, cat # 33069-62-4 ), DOXO (Sigma-
Aldrich, cat # 25316-40-9) and CBZ (Sanofi-Aventis, provided by collaborator). TNF-related apoptosis inducing ligand (TRAIL) was purchased from Peprotech and Actinomycin D was purchased from R&D Systems. The broad caspase inhibitor z-VAD-fmk was obtained from Enzo Life Sciences. Necrostatin, a RIP-3 inhibitor, and Necrosulfonamide, a MLKL inhibitor, were obtained from Sigma-Aldrich (cat # N9037-10MG) and Millipore (cat# 480073-25MG) respectively.

Since the experiments in which we induced cell death in the presence and absence of inhibitors of apoptosis and necroptosis were not part of our published work, the stock and effective concentrations were as follows:

- Broad caspase inhibitor zVAD-fmk: Stock 20mM and effective concentrations of 10μM and 20μM. Pre-treat cells for 1 hour before adding other drugs.
- Necrostatin: Stock 20mM and effective concentration of 30μM. Pre-treat for 1 hour.
- Necrosulfonamide: Stock 10mM and effective concentration of 10μM. Pre-treat for 2 hours.

**Assessment of Cell Viability**

Cell morphology was visualized on an Olympus IX70 microscope equipped with Hoffmann Modulation Contrast (Olympus American) and a digital Spot Imaging System (Diagnostic Instruments). To assess viability, cells were seeded in 96-well plates at a density of 1x10^4 cells per well and then treated with each individual drug in at least triplicates. A modified 3-(4,5-dimethylthiazol- 2-yl)-2,5- diphenyltetrazolium bromide
(MTT) assay (Sigma-Aldrich) was performed as described previously by Mediavilla et al, 2009 [3]. Briefly, 5mg of MTT reagent were dissolved per 1ml of PBS, to a final concentration of 200mg of MTT reagent to 20ml of PBS. The suspension needs to be vortexed until all solid is dissolved. Since this reagent is light-sensitive, experiments were performed in the tissue-culture hood with the lights off and the tube containing the MTT reagent was protected with foil. Then, we added 25μl of MTT suspension to each well in a 96-well plate where cells are growing in monolayer followed by incubation at 37°C in the tissue culture incubator for 2 hrs. After incubation, plates were spun at 4000 rpm for 5 minutes to keep the cells attached to the bottom of the plate. This modification to the MTT protocol was done on all assays involving taxanes since these agents are microtubule-stabilizing drugs, causing cells to round-up and lose their attachment to the plate surface even if when they are still viable. Then, media containing MTT was discarded carefully by flicking the plate first on the sink and then carefully on napkin. Media aspiration can also be done, but it must be done carefully since the cells could detach from the surface. We then added 300μl of DMSO to each well using a multi-pipettor and rocked for about 10 minutes until the MTT formazan substrate was dissolved. Absorbance was measured at 450 nm using a μQuant microplate reader (Bio-tek Instruments) or a SpectraMax reader. Values were normalized to the absorbance obtained for the untreated control cells. The approximate drug half-maximal effective concentration (EC50) was determined using the plotted values of the dose response curve. Each value represented the mean value of at least three different independent experiments performed in at least triplicate samples. The standard error of the mean (SEM) was calculated for each value.
Immunoblotting Procedures

The following commercially acquired antibodies were used in our studies: rabbit polyclonal anti-LEDGF/p75 (1:1000, Bethyl Laboratories Inc. catalog# A300-848A); rabbit monoclonal anti-β-actin (1:5000, Cell Signaling catalog # 5125); rabbit polyclonal anti-cleaved caspase-3 (1:1000, Cell Signaling catalog # 9661); rabbit monoclonal anti-P-gp/MDR1/ABCB1 (1:1000, Cell Signaling catalog # 13342) and horseradish peroxidase (HRP)-labeled secondary IgG antibodies (goat anti-rabbit IgG, 1:5000, Thermo Fisher Scientific catalog # 31466; goat anti- human IgG/IgA/IgM, 1:5000, Thermo Fisher Scientific catalog # A18847). The human serum autoantibody to LEDGF/p75 (initially diluted at 1:200 but could be used at higher dilution depending on titer) was from the serum bank of the Casiano Laboratory at the Center for Health Disparities and Molecular Medicine at Loma Linda University School of Medicine.

Whole cell lysates were prepared as follows. To collect cells, we detached them from the surface of tissue culture flasks or plates using 200μl of trypsin or by adding 1ml of PBS and scraping the cells with cell scraper. Cell suspensions were collected in 5 ml plastic tissue culture tubes and stored in ice. We then spun the cells for 1 minute in a clinical centrifuge at 4,000rpm, discarded the supernatant carefully, and washed the pellet with PBS by additional centrifugations. This step was repeated 3 times to eliminate any residual trypsin and media. This is important because albumin is highly abundant in medium and may interfere with electrophoresis and immunoblotting if not adequately removed. We dissolved the pellet in Laemmli sample buffer containing a protease inhibitor cocktail (LSB:CPI, volume depends on size of pellet but typically we started with 100μl of this reagent for cells collected from two 6-well plates seeded at 120,000
cells per well). Cells were sonicated on ice to disrupt cellular structures. The lysates were stored at -80°C for up to 1 year to preserve protein integrity.

Immunoblotting was performed following this procedure. Equal amounts of protein from whole cell lysates were loaded into individual wells of gels (SDS-PAGE, NuPAGE). Depending on the proteins to be separated we used a variety of gels, such as 4-12% (most commonly used) and 4-20% polyacrylamide gradient gels, or fixed 10% and 12% polyacrylamide gels. The latter have a 4% stacking gel, which allows for sharper protein bands as they enter the 10% or 12% separating gels. All gels were obtained from Thermo Fisher Scientific (previously Invitrogen and then Life Technologies). Protein concentration was determined utilizing the DC protein assay kit from BioRad following the manufacturers instructions. To observe and compare changes in protein expression between cell lines, only 5μg-10μg should be loaded onto wells to avoid saturating the chemiluminescence signal in immunoblots. For other applications, 20μg to 40μg is ideal but the sensitivity of the antibody should also be considered.

Proteins were separated by SDS-PAGE and transferred into polyvinyl difluoride (PVDF) membranes (Millipore) in a NuPAGE electrophoresis system by Thermo Fisher Scientific. To prepare the samples after calculating the desired protein concentration, we diluted the appropriate protein volume in 2.5μl NuPAGE LDS sample buffer (4X) and 1μl of NuPAGE reducing agent (10X). Final volume, typically 10μl, was achieved utilizing deonized water.

The buffers utilized were purchased from the manufacturer. The protein separation was done in MOPS SDS running buffer for 60 minutes at 175 volts. The protein transfer was done in transfer buffer diluted to a concentration of 1X (the buffer
comes in a 20X concentration) with 10% methanol and 1ml of antioxidant for 90 minutes at 25 volts.

Membranes were blocked in 5% dry milk or 5% Bovine Serum Albumin (BSA), depending on the primary antibody, prepared in TBS-T buffer (20 mM Tris- HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). Typically, we blocked for 2 hours when utilizing commercial antibodies. Blocking was done rocking slowly in the cold room. Membranes were then probed individually with primary and corresponding secondary antibodies and washed 3 times for 10 minutes with TBS-T between each antibody application. The incubation time varies between antibodies and should be done following manufacturer’s instructions. When utilizing a rabbit anti-LEDGF/p75 antibody by the company Bethyl (cat#: A300-848A), we incubated with primary antibody at a 1:1000 dilution overnight rocking in the cold room. We then washed with TBS-T 3 times, changing TBS-T every 10 minutes. Then, we applied the appropriate secondary antibody for 2 hours in a wet chamber. After incubation, we repeated the washing cycles.

It is important to follow the approved safety protocol when using human sera to probe membranes. First, protective goggles, gloves, and lab coat must be worn at all times. Protective and absorbent cover should be placed over the bench to ensure that if any serum is spilled, it does not contaminate the work area. Pipet tips that were used to pipet human serum should be place in a container with bleach to de-contaminate them before discarding in approved container.

We preferably thawed human sera by placing the tubes in ice a few hours before use. If sera must be thaw immediately, put the tube in water bath but return to ice immediately after thawing. If possible, aliquot serum samples to minimize thawing and
freezing cycles, which may inactivate serum autoantibodies and other proteins. Serum reactivity will vary from vial to vial depending on the titer of the autoantibodies present. If the autoantibody titer is high or if the vial has been thawed and frozen multiple times, you might get excessive “background” in the chemiluminescence signals, which will impede the visualization of the protein bands. To minimize this, we blocked the membranes to be exposed to human serum in TBS-T with 0.1% Tween and 5% milk for 2 hours. Albumin was not used in blocking solution when human sera was used due to the presence of anti-albumin autoantibodies in human serum, which will increase the background signal. We applied primary human serum autoantibody at a 1:200 dilution in TBS-T with 5% milk (serum dilution depends on the titer of the particular autoantibody) to the membranes placed on top of paraffin in a wet-chamber for 2 hrs. This was done to use the minimum amount of serum possible for those sera available in limited volumes. Alternatively, for high titer autoantibodies for which we had large serum volumes, membranes were placed in small plastic trays and incubated at room temperature with the diluted serum in a rocking platform, with the rocking at the lowest setting possible to minimize background formation. After primary serum antibody application, the membrane was washed at least 5-7 times, changing media every 10 minutes, and placed in a wash plastic tray in the rocking platform, with minimum rocking. For each wash, we held the membrane with tweezers and washed it (both sides) from top to bottom with TBS-T (no milk) following a zig-zag pattern using a wash bottle with spout. We then incubated the membrane with appropriate secondary HRP-labeled rabbit- or goat-anti-human antibody at a 1:1000 dilution (TBS-T with 5% milk) for 1-2 hours in the wet chamber or plastic tray. Again, we washed at least 5-7 times, changing
media every 10 minutes, rocking at the lowest setting.

Enhanced chemiluminescence (ECL) was used to detect immunoreactive protein bands. For this, the ECL Western Blotting Substrate (Thermo Fisher Scientific Pierce, catalog # 32106) was added to the antibody-protein surface of each PVDF membrane, followed by incubation for 5 minutes. Membranes were then transferred to autoradiography cassettes and exposed to autoradiography films for different lengths of time to ensure accurate detection of immunoreactive protein bands.

**Indirect Immunofluorescence Microscopy**

To visualize endogenous LEDGF/p75 expression, PCa cells were grown as monolayer in chamber slides until they reached a confluency of 70%. The media was carefully aspirated and cells were then washed twice with PBS by carefully spraying at a corner of the chamber wells with a wash bottle with spout and aspirating the liquid without touching the cells. Cells where then fixed with 4% formaldehyde for 20 minutes and permeabilized with 0.5% Triton X-100 for 10 minutes. Cells were then incubated with human anti-LEDGF/p75 serum autoantibody at 1:200 dilution for 1 hr in a humid chamber. After washes with PBS, cells were incubated with an FITC-conjugated goat anti-human IgG (H+L) secondary antibody (1:200, Thermo Fisher Scientific catalog # 62-7111), used at 1:200 dilution. Chamber wells were removed carefully, including any residual glue, with tweezers. Cells were counterstained with medium containing 4’,6-diamidino-2- phenylindole (DAPI) and an anti-fade (Vectashield), and a coverslip was then carefully placed over the cells. To remove excess medium and bubbles, we placed a kim-wipe on top of the coverslip and pressed carefully from the top avoiding lateral
movements to prevent dislodging the cell monolayer. As an alternative to chamber slides some experiments were conducted in commercially available ANA slides (acquired as a kit from Inova Diagnostics), which already have HEP-2 (HeLa derivative) cells grown in monolayer and fixed/permeabilized in individual wells. The procedures were similar but followed the specifications of the manufacturer.

Images were acquired in a BioRevo Keyence BZ-9000 fluorescent microscope (Keyence). Alternatively, high-resolution images can be acquired using a confocal microscope at the LLU Advanced Imaging Facility. It is critical that all images corresponding to a particular antibody and fluorescent probe should be obtained using the same parameters, particularly if expression levels of the protein of interest are been assessed in different cell lines or under different treatments or conditions.

**RNA Interference-Mediated Knockdown of LEDGF/p75 in PCa Cells**

To achieve transient knockdown of LEDGF/p75 in our cellular models, the si-LEDGF/p75 sequence corresponded to nucleotide sequence 1340–1360 (‘5-AGACAGC AUGAGGAAGCGAdTdT-3’), present in a C-terminal region of LEDGF/p75 that is not shared by its short alternative splice variant LEDGF/p52. This was critical to ensure specific knockdown of LEDGF/p75, leaving LEDGF/p52 intact. Cells were transfected with 100nM siRNAs or up to 500nM depending on endogenous LEDGF/p75 levels, using oligofectamine (Life Technologies) following manufacturer’s instructions. A scrambled siRNA duplex (siSD, Dharmacon) was used as a negative control at a concentration of 50nM. To seed the cells we used antibiotic free media, which ensure that antibiotics do not interfere with the uptake of the siRNA oligos. We also prepared the
oligofectamine-siRNA complexes in serum free media since serum interferes with the formation of these complexes. Based on our experience [1], LEDGF/p75 levels remain depleted for up to 96 hrs, after transfection as determined by immunoblotting, however, this may vary depending on the growth rate of the particular cell line used in the experiment.

Clonogenic Assays

Cells with and without LEDGF/p75 knockdown were cultured at a density of 1x10^3 cells per well in 6-well plates and treated with increasing concentrations of drugs as described previously. Since 1x10^3 per well is a relatively small number of cells and consequently there may be problems with attachment due to lack of cell-cell contact, it is important to remove all residual trypsin by washing the cells 2-3 times with PBS after trypsinization. For this, we collected cells in a 5ml tube, centrifuged them at 4,000rpm for 2 minutes, and discarded the supernatant. We resuspended the cells gently in 2-5ml of media to make sure that the final volume of the cell suspension to be added to each well was not less than 1μl. This final volume depends on the number of live cells per ml in your cell suspension. This must be used to calculate the volume of cells to be seeded in a final volume of 2 ml of media per well in 6-well plates. Also, we treated the plated cells with each drug when they were already fully attached to the bottom surface of the wells and not round. Thus, we waited at least overnight to ensure proper cell attachment.

Cells were grown for 12 days, which is typically when the surviving colonies were visible. After removing the media, the colonies were washed with PBS by gently swirling or rocking for 5 minutes with enough PBS to cover the wells. We then added
are 1ml of fixing solution (3:1 methanol:acetic acid) to each well and rocked or swirled for 20 minutes. The fixing solution was carefully aspirated and colonies were gently washed twice with PBS. Colonies were stained with 0.5% crystal violet diluted in methanol for 10 minutes. Finally, crystal violet solution was removed by washing with double distilled water in a sink (carefully without directly spraying the colonies). Plates were inverted on top of paper towel and dried overnight.

Images of each individual plate were acquired using the VisionWorks Acquisition and Analysis software in a GelDoc-It² imager (UVP, Analytik Jena Company). The Automated Colony Counting capability of the software was used to count each colony in the individual wells using the same parameters for each plate. At least three plates from three independent experiments were used for quantification of colonies under a particular experimental condition or treatment. In addition to number of colonies, we should also consider changes in colony size as a parameter of cellular proliferation in response to drug treatments.

**Statistical Analyses**

The Student’s t-test (paired, two-tailed) was used to evaluate differences between treatment and control groups using Microsoft Excel. Differences were considered statistically significant at P values equal or below 0.05. As a side note, a paired student T-test should not be used when comparing multiple parameters due to the introduction of significant errors. For this, use a two-tailed t-test or another statistical method considering each of their assumptions.
Additional protocols and experimental details can be found in the laboratory notebook and page numbers as indicated in Table 2 below. These notebooks are stored in the Casiano Laboratory, located in the first floor of Mortensen Hall in Loma Linda University, Loma Linda CA. All electronic files pertaining this dissertation work are stored in the Casiano laboratory computers and the Casiano Laboratory external drive memory under the folder Leslimar Rios Colon Casiano Lab Files
**Table 2:** Location of protocols and experimental details in Leslimar Rios-Colon’s laboratory notebooks.

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<tr>
<th>Protocol</th>
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References


