Vesicular stomatitis virus and BCL-2 inhibitor combination therapy for the treatment of chronic lymphocytic leukemia

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

by

Sara Samuel

Department of Microbiology and Immunology, McGill University, Montreal

March 2013

Abstract

Chronic lymphocytic leukemia (CLL) is a cancer of the white blood cells (B cell lymphocytes). It is an indolent disorder that results in the accumulation of CD5+ B cells. In CLL, resistance to cell death is attributed to the overexpression of several key pro-survival proteins (i.e. B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia- 1 (Mcl-1)) that belong to the apoptotic Bcl-2 family of proteins. Bcl-2 and Mcl-1 overexpression deregulates both the apoptotic and autophagic signaling pathways and contributes to tumorigenesis.

Oncolytic virotherapy has emerged as a novel anti-cancer therapy for the treatment of a variety of malignant disorders. Oncolytic virus (OV) Vesicular stomatitis virus (VSV)-AV1 takes advantage of genetic defects present within cancerous cells and preferentially targets and kills them. Normal cells are spared the cytotoxic effects of viral lysis and are left unharmed. CLL cells are largely resistant to VSV oncolysis due to an elevated protein expression level of Bcl-2 and Mcl-1 as well as the inhibitory interactions Bcl-2 and Mcl-1 form with pro-apoptotic and pro-autophagic proteins. It is a common approach to use combination therapy to overcome limitations with single agent treatment. In the studies presented within, VSV-AV1 treatment was combined with small-molecule Bcl-2 inhibitors as a means to overcome resistance to oncolytic virtotherapy observed in CLL patients.

In the first strategic approach, we combined low-dose amounts of the pan–Bcl-2 inhibitor, Obatoclax, with VSV-AV1 and examined the effect on the apoptotic signaling pathway. Obatoclax and VSV-AV1 synergistically enhanced cell death in primary CLL cells. The combination therapy induced intrinsic apoptotic signaling through the activation of caspases-3 and -9 cleavage. Inhibitory complexes between Bcl-2:Bax and Mcl-1:Bak were disrupted as well.

Pro-death protein, Noxa, was upregulated following VSV-AV1 infection and as identified as a critical mediator of apoptotic cell death.

.

In the second approach, we examined VSV-AV1 virotherapy in combination with Bcl-2 inhibitors (Obatolcax or ABT-737) to elucidate the role of the autophagic pathway on cell death in primary CLL cells. We also investigated the crosstalk between the autophagic and apoptotic pathways following combination treatment. Bcl-2 inhibitor/VSV-AV1 therapy led to increased LC3-II and reduced p62 proteins levels, which signify the activation of autophagy. Inhibition of autophagy, with 3-methyladenine, significantly increased apoptotic cell death induced by Bcl-2 inhibitor/VSV-AV1 treatment. The combination therapy also abrogated Bcl-2:Beclin interactions thus stimulating the induction of autophagy.

Altogether, our therapeutic strategies indicate that Bcl-2 inhibitors improve VSV-AV1 oncolysis in treatment-resistant hematological malignancies, such as CLL, with characterized defects in apoptotic and autophagic responses.

Résumé

La leucémie lymphoïde chronique (LLC) est un cancer qui affecte les globules blancs et provient de l'accumulation des lymphocytes B CD5+. La résistance à la mort cellulaire est attribuée à la surexpression de plusieurs protéines de pro-survie tel que « B-cell lymphoma 2 » (Bcl-2) et « myeloid cell leukemia (Mcl-1) » qui appartiennent à la famille de protéines apoptotiques Bcl-2. La surexpression de Bcl-2 et Mcl-1 dérégule les voies de signalisations apoptotiques et autophagiques et contribue au développement de la tumeur.

La virothérapie oncolytique a démontré son efficacité comme nouvelle thérapie anticancéreuse pour le traitement de plusieurs types de tumeur. Le virus de la stomatite vésiculaire (VSV)-AV1 est un virus oncolytique qui prend avantage des anomalies génétiques présentes dans les cellules cancéreuses pour préférentiellement infecter et détruire ces dernières tandis que les cellules normales sont épargnées de la lyse virale. Cependant, les cellules affectées par LLC sont tout de même résistante à l'oncolyse par VSV à cause d'un niveau d'expression élevé des protéines Bcl-2 et Mcl-1, et de leur effet inhibiteur sur les protéines pro-apoptotiques et pro-autophagiques. Les thérapies combinées permettent de dépasser les limites imposées par les traitements utilisant un seul agent anticancéreux. Dans la présente étude, le traitement oncolytique VSV-AV1 a été combiné avec un autre agent anticancéreux, une molécule inhibitrice de Bcl-2, pour surpasser la résistance au traitement oncolytique par VSV-AV1.

Dans une première stratégie, nous avons combiné une faible dose d'Obatoclax, un inhibiteur de Bcl-2, avec VSV-AV1 et examiné l'effet sur la voie de signalisation apoptotique. Obatoclax et VSV-AV1 ont augmenté de façon synergique la mort cellulaire des cellules primaires isolées de patients atteints de LLC. La thérapie combinée a induit la signalisation apoptotique intrinsèque en activant la caspase-3 et caspase-9 ainsi qu'en séparant les complexes inhibiteurs

Bcl-2:Bax et Mcl-1:Bax. De plus, l'expression de la protéine pro-apoptotique Noxa a augmenté suite à l'infection avec VSV-AV1 et il a été démontré que cet événement est critique pour enclencher la mort cellulaire par apoptose.

Dans une deuxième stratégie, nous avons examiné le rôle de la voie de signalisation de l'autophagie dans la mort cellulaire des cellules primaire LLC suite à la thérapie oncolytique avec VSV-AV1 en combinaison avec Obatoclax ou ABT-737, deux inhibiteurs de Bcl-2. Nous avons étudié l'interaction entre l'autophagie et l'apoptose suite au traitement combiné et démontré que le traitement a augmenté le niveau de LC3-II et a réduit le niveau de la protéine p62, ce qui indique une activation de l'autophagie. En contrepartie, l'inhibition de l'autophagie avec le 3-methyladénine a augmenté de façon significative la voie apoptotique. La thérapie combinée a également bloqué l'interaction entre Bcl-2 et Bectin et par conséquent stimulé l'induction de l'autophagie.

Les stratégies thérapeutiques examinées dans cette étude indiquent que les inhibiteurs de Bcl-2 améliorent l'oncolyse virale dans les leucémies résistantes aux traitements simples et qui sont caractérisées par des anomalies dans la réponse apoptotique et autophagique, tel qu'observé chez les patients atteints de LLC.

Acknowledgements

It is with immense gratitude that I acknowledge the support and help of my supervisor **Dr. John Hiscott.** I am most grateful for your mentorship, guidance and support throughout my research and thesis.

I am thankful to my co-supervisor **Dr. Rongtuan Lin** for your support, insight and encouragement.

Thank you to Dr. Wilson Miller for your helpful contributions as part of my Ph.D. advisory committee.

I would like to thank both current and past members of the Hiscott and Lin lab for the many helpful discussions, encouragement and assistance throughout my research work. A special thanks to Vanessa Tumilasci for all your great help.

It is a great pleasure to thank, Suzanne Paz, Kemba Mulrain, Marie-Line Goulet and Fethia Ben Yebdri, all of whom helped me write and edit my thesis successfully.

Lastly, thank you to my family, especially my mother for her moral support, and my dear friends for their patience and understanding.

Preface

In accordance with the "Guidelines for Thesis Preparation", this thesis is presented in a Manuscript-based format. A general introduction precedes the results chapter which is divided into two sections:

1- Manuscript I

Samuel S, Tumilasci VF, Oliere S, Nguyên TL, Shamy A, Bell J, Hiscott J. **VSV Oncolysis in Combination With the BCL-2 Inhibitor Obatoclax Overcomes Apoptosis Resistance in Chronic Lymphocytic Leukemia**. Mol Ther. 2010 Dec;18(12):2094-103.

2- Manuscript II

Samuel S, Beljanski V, Van Grevenynghe J, Richards S, Ben Yebdri F, He Z, Nichols C, Belgnaoui S.M, Steel C, Goulet M, Shamy A, Brown D, Abesada G, Haddad EK, Hiscott J. **BCL-2 inhibitors sensitize therapy-resistant chronic lymphocytic leukemia cells to VSV oncolysis.** Mol Ther. 2013 May 21. doi: 10.1038/mt.2013.91

Contributions of the authors

Manuscript I: SS and VFT conceived, designed and performed the experiments, and wrote the manuscript. SO performed experiments, TLN edited the manuscript, AS provided cohort of CLL patients, JB provided VSV strains used in this work and JH conceived experiments, wrote the manuscript and supervised study.

Manuscript II: SS conceived, designed and performed the experiments, and wrote the manuscript. VB analyzed microarray data and wrote the manuscript, JVG designed and performed experiment, SR, FBY, ZH, CN, SMB and MG performed experiments, CS performed microarray experiments and data anslysis, AS, DB and GA provided cohort of CLL patients, EKH revised the manuscript and JH conceived experiments, wrote the manuscript and supervised study.

In addition collaborative work with laboratory members resulted in the following co-authored publications:

Leveille S, Samuel S, Goulet ML, Hiscott J. **Enhancing VSV oncolytic activity with an improved cytosine deaminase suicide gene strategy.** Cancer Gene Ther. 2011 Jun;18(6):435-43.

Belgnaoui SM, Paz S, Samuel S, Goulet ML, Sun Q, Kikkert M, Iwai K, Dikic I, Hiscott J, Lin R. **Linear ubiquitination of NEMO negatively regulates the interferon antiviral response through disruption of the MAVS-TRAF3 complex**. Cell Host Microbe. 2012 Aug 16;12(2):211-22.

Table of Contents

List of Figures and Tables

CHAPTER 1 – INTRODUCTION

CHAPTER 2 – RESULTS

Manuscript I- VSV Oncolysis in Combination With the BCL-2 Inhibitor Obatoclax Overcomes Apoptosis Resistance in Chronic Lymphocytic Leukemia

Manuscript II- BCL-2 inhibitors sensitize therapy-resistant chronic lymphocytic leukemia cells to VSV oncolysis

CHAPTER 4 - CONCLUDING REMARKS AND CONTRIBUTION TO ORIGINAL KNOWLEDGE

List of Abbreviations

3-MA	3-Methyladenine
A.k.a.	also known as
Aa	Amino Acid
Abs	Antibodies
Ad	Adenovirus
ADP	Adenosine diphosphate
AIF	Apoptosis-Inducing Factor
Alpha	α
AMPK	AMP-activated protein kinase
APAF-1	Apoptotic protease activating factor-1
Apogossypol	ApoG2
ASCT	Autologous haematopoietic stem cell transplantation
ATG	Autophagy-related genes
ATL	Adult T-cell leukemia
ATM	Ataxia eleangiectasia mutated
ATP	Adenosine-5'-triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	BCL-2 antagonist or killer
Bax	Bcl-2-associated X protein
Bcl	B-cell lymphoma
Bcl-x_L	Bcl-extra long
BCR	B- cell receptor
Beta	β
BH	Bcl-2 homolgyhomology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
Bmf	Bcl-2 modifying factor
Boo	Bcl-2 homolog of ovary

Box	Bcl-2 related ovarian killer
CARD	Caspase recruitment domain
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CLL	Chronic lymphocytic leukemia
COG	Clusters of orthologous groups
CQ	Chloroquine
CRD	Cysteine-rich extracellular domain
DC	Dendritic cells
DD	Death domains
DED	Death effector domain
DIABLO	Direct inhibitors of apoptosis protein with low pI
DIOC6(3)	3,3'-dihexyloxacarbocyanine iodide
DISC	Death-inducing signaling complex
DR	Death receptors
DRAM	Damage-regulated autophagy modulator
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FADD	Fas-associated death domain
FasL	Fas Ligand
FIP200	focal adhesion kinase family interacting protein of 200
FISH	Fluorescence in situ hybridization
FoxO	Forkhead box O
G	Glycoprotein
Gamma	γ
GAS	IFNgamma activated site
GCN2	general control nonrepressor 2
HLA	Human leukocyte antigen
Hrk	Hara-kiri
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS)

Hsp90	Heat shock protein 90
HSV	Herpes Simplex Virus
HTLV	Human T-cell lymphotrophic virus
IAP	Inhibitors of Apoptosis
IB	Immunoblotting
IFN	Interferon
IFNAR	IFN alpha/beta Receptor
IFNGR	IFN-gamma receptor
Ig	Immunoglobulin
IgVH	Immunoglobulin heavy-chain variable region
IL	Interleukin
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP	Immunoprecipitated
IPS-1	IFN-β promoter stimulator 1
IRF	IFN-regulatory factor
ISGs	IFN-stimulated genes
JAK	Janus Kinase
KO	Knockout
L	Large polymerase protein
LAMP	Lysosomal-associated membrane protein
LC3 (MAP-LC3)	Microtubule-associated protein light chain 3
M	Molar
M	Methionine
M	Matrix protein
mAb	Monoclonal antibody
MAC	Mitochondrial apoptosis-induced channel
Mcl-1	Myeloid cell leukemia factor -1
MEFs	Mouse embryonic fibroblasts
Mfn	Mitofusins
MHC	Major histocompatibility complex

Micro	μ
miR	micro-RNA
MOMP	Mitochondrial outer membrane permeabilization
mTOR	mammalian target of rapamycin
MV	Measles virus
N	Nucleocapsid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIS	Sodium iodine symporter
NK	Natural killer
NMR	Nuclear magnetic resonance
NOD	Nucleotide-binding and oligormerization domain
NSCLC	Nonsmall cell lung cancer
OMM	Outer mitochondrial membrane
OPA1	Optic Atrophy 1
OV	Oncolytic virus
P	Phosphoprotein
PAS	Pre-autophagosomal structure
PBMCs	Peripheral blood mononuclear cells
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PIDDosome	p53-induced protein with a death domain
Poly(A)	Polyadenylated
PS	Phosphatidylserine
PSA	Prostate-specific antigen
PtdIns(3)P	Phosphatidylinositol 3-phosphate
Puma	promoter upregulated modulator of apoptosis
R	Arginine
Rab	Ras-related GTP-binding protein
RAIDD	RIP-associated Ich-1/Ced-3 homologous protein with a death domain

RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid–inducible gene I
RNP	Ribonucleoprotein
Rubicon	Run domain protein as Beclin 1 interacting and cysteine-rich containing
SAR	Structure-activity relationship
SCID	Severe combined immunodeficient
SCLC	Small cell lung cancer
siRNA	small-interfering RNA
SLAM	Signaling lymphocyte-activating molecule
SMAC	Second mitochondrial activator of caspases
SMH	Somatic hypermutation
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
ss	Single stranded
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
tBid	truncated Bid
TCR	T- cell receptor
TM	Transmembrane
TNF	Tumor necrosis factor
TNFR1	Type I TNF receptor
TP53	Tumor protein 53
TRADD	TNF receptor–associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
Tyk	Tyrosine kinase
U	Uracil
ULK	Unc-51-like kinase
Unc	Uncoordinated
UVRAG	UV irradiation resistance-associated gene
VAMP	Vesicle-associated membrane protein

v-ATPases	Vacoular ATPases
VDAC	Voltage-dependent anion channel
VDV-NJ	Vesicular stomatitis virus New Jersey strain
Vps	Vacuolar protein sorting
VSV	Vesicular stomatitis virus
VSV-Ind	Vesicular stomatitis virus Indiana strain
Wt	Wildtype
ZAP-70	Zeta-associated protein 70

CHAPTER 1

INTRODUCTION

1.1 Oncolytic Viruses (OVs)

Oncolytic virotherapy is a novel treatment employing cancer-specific viruses to destroy cancer cells (Russell *et al.*, 2012). It is quite fascinating to think that a virus can be used for such a cause, but once you understand the nature of a virus, one can easily see how they can benefit cancer therapy. Viruses are small infectious agents, consisting of nucleic acid (DNA or RNA genome) enclosed within a protein coat or capsid (Cann, 2011; Lodish H, 2000).Viruses are obligate parasites that can only replicate within the cells of other organisms; they infect a wide host range and many cell types. There are two types of viral reproduction: lytic and lysogenic. During the lytic life cycle, viruses enter host cells, replicate, release progeny by lysis (cell death), and spread to other host cells. As part of the lysogenic cycle, viruses incorporate their nucleic acid into the host genome and remain latent; viral replication occurs when the host cell divides (Akao *et al.*, 1994). The pathogenic properties of lytic viruses make them ideal candidates for oncolytic virotherapy. There is a long history demonstrating the anti-cancer activity of viruses (Kelly *et al.*, 2007). Leukemia and lymphoma patients often exhibit signs of cancer regression following naturally acquired contraction of influenza or measles virus. The therapeutic properties of viruses were gradually recognized by the 20th century and clinical trials testing oncolytic viruses (OVs) have been carried out since the late 1940s. The field has expanded substantially over the past few decades, and great strides have been made in the understanding of virology and the exploitation of viruses as cancer-fighting agents.

Cancer cells are malignant cells that grow uncontrollably and are resistant to cell death (Cooper, 2000). Several mechanisms of cell destruction, apoptosis, autophagy, and necrosis are blocked in cancer cells and this facilitates tumor survival (Elmore, 2007; Hanahan *et al.*, 2000; Ouyang *et al.*, 2012; Pattingre *et al.*, 2006). Cancers also utilize several biological strategies to evade recognition by the immune

system as a means of survival (Critchley-Thorne *et al.*, 2009; Mapara *et al.*, 2004). This unique intrinsic biology also explains their susceptibility to OV infection and cell killing. Many cancers are defective in interferon (IFN) signaling, which results in diminished anti-tumor innate and adaptive immune responses and supports tumor evasion. IFN signaling mediates a number of p53-regulated anti-tumor and antiviral responses (Takaoka *et al.*, 2003). p53 is a tumor suppressor protein that has a functional role in cell cycle arrest and apoptosis. Mutations in the p53 gene are very common in human cancers (Hollstein *et al.*, 1991). Overactivation of Ras signaling — regulator of cell proliferation, survival, and differentiation — is also frequently found in cancers (Downward, 2003). OVs exploit many of these evasion and survival mechanisms to preferentially infect and lyse cancer cells because most of these mechanisms create environments that favour viral replication.

1.1.1 Properties of OVs

Many naturally occurring viruses and genetically engineered viruses have been used for oncolytic virotherapy:Vesicular stomatitis virus (VSV), Reovirus, Newcastle disease virus, Measles virus (MV), Adenovirus (Ad), Vaccinia virus and Herpes simplex virus (HSV), to name a few. An ideal OV should display several of the following characteristics (Parato *et al.*, 2005);

1) Preferential targeting and lysing of cancer cells
2) Broad spectrum of tissue and cancer tropism
3) Sensitivity to host antiviral response initiated in normal cells
4) Limited adverse effects and toxicity in normal cells
5) Cytoplasmic replication and inability to incorporate into the host cell genome
6) Rapid replication, lysis and viral spread
7) Proficient delivery to tumors through a systemic route

There is a long list of prototypical OVs that are currently being studied and tested in pre-clinical and clinical models; the following section is by no means an exhaustive list of these viruses. VSV is the main focus of this project and is therefore described in greater detail.

1.1.2 Vesicular Stomatitis Virus (VSV)

VSV is a single-stranded (ss) RNA virus belonging to the *Rhabdoviridae* family (Rodriguez L.L., 1999). In Greek, rhabdos = rod, referring to the rod/bullet shape of the inclusive viruses. Rhabdoviruses typically infect vertebrates (i.e. humans, cattle, pigs, and horses) and plants, and are transmitted via arthropod vectors (i.e. sandflies, mosquitoes) and plant tissues. Of the all the Rhabdoviruses, VSV is the most extensively studied. VSV is a member of the genus *Vesiculovirus*, in the family *Rhabdoviridae*, and of the order *Mononegavirales*. VSV infection is the underlying cause of vesicular stomatitis disease. During disease progression, animals develop vesicular lesions in the mouth, tongue, udder teats and hoof (Rodriguez L.L., 1999). Two main serotypes of VSV have been defined: VSV Indiana strain (VSV-Ind) and VSV New Jersey strain (VSV-NJ). Both viruses were isolated from outbreaks in cattle in the 1920s; VSV-Ind is the most studied strain (Whelan, 2008). Insect vectors spread VSV to animals, but natural infection in humans is extremely rare (Roberts *et al.*, 1999). VSV infection in humans is primary through contact with infected livestock. Additionally, laboratory personnel who are frequently exposed to the virus may become infected. The seroprevalence of antibodies against VSV within the general population is very low (Roberts *et al.*, 1999). For the most part, humans who acquire VSV infection do not experience any noticeable symptoms. A few infected patients display fever-like symptoms, intermittent chills, myalgia and nausea; fever and all other acute symptoms resolve within 3 days (Johnson *et al.*, 1966; Roberts *et al.*, 1999). An isolated case of encephalitis (acute inflammation of the brain) was reported in a 3 year old patient who was infected with VSV(Quiroz *et al.*, 1988).

1.1.2.1 Virion

VSV particles are ~180 nM in length and ~70 nm in diameter (Whelan, 2008) (Figure 1). The virion particles are 74% protein, 20% lipid, 3% RNA, and 3% carbohydrate material. The viral envelope is composed of trimeric glycoprotein (G) (67 kDa). The viral core contains a ribonucleoprotein (RNP) structure consisting of the RNA genome, which is complexed with the nucleocaspid (N) protein. The large (L) polymerase protein and the phosphoprotein (P) form the RNA-dependent RNA polymerase (RdRp). The RNP structure and the RdRp collectively form a helical nucleocapsid structure. The virus also contains a peripheral matrix (M) protein that binds the RNP structure and membrane G protein. The ratio of molecules in a single VSV particle is 1RNA:1200N:500P:1800M:1200G:50L.

1.1.2.2 Genome

The VSV genome is an 11-kilobase long, nonsegmented, ss, negative-sense RNA (Rodriguez L.L., 1999). The genomic RNA commences with a 3' hydroxyl leader sequence, followed by mRNA encoding all five proteins (G, N, L, P and M), and terminates with a 5' phosphate group (Rodriguez *et al.*, 1997; Whelan, 2008). No modifications (i.e. 5' end cap and 3' end poly(A) tail) are made to the genome ends; however, the individual mRNA encoding the 5 viral proteins are capped and polyadenylated (Ball *et al.*, 1999). Viral infection is dependent on the RNA genomes' association with the nucleocaspid protein; therefore, naked RNA alone is unable to drive the replication cycle and generate infectious particles (Whelan, 2008).

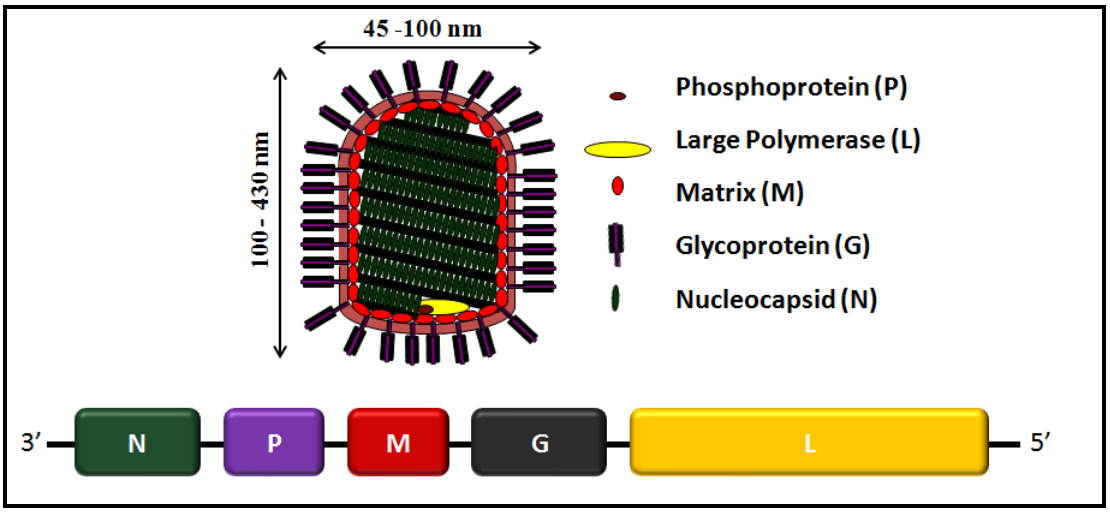

Figure 1: Schematic diagram showing VSV structure and genome.

VSV is a bullet-shaped virus particle. The VSV genome encodes five major proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large polymerase protein (L).

1.1.2.3 Replication Cycle

Viral infection begins with VSV G protein binding to a still unknown surface receptor of the host cell (Whelan, 2008) (Figure 2). Phosphatidylserine (PS), a membrane phospholipid, was long believed to be the host cell receptor responsible for VSV binding. However, Coil *et al* have disproved this hypothesis (Coil *et al.*, 2004). It is clear that the actual receptor is either ubiquitously expressed, as the virus infects several cell types, or that the VSV G can bind to a number of molecules to initiate viral attachment (Carneiro *et al.*, 2002). Nevertheless, once the virus binds, its entry is facilitated by an actin and clathrin-mediated endocytosis mechanism, where it is transported to early endosomal compartments (Cureton *et al.*, 2010). Endosomal acidification triggers the fusion of the VSV envelope and the endosome membrane. Changes in pH levels inhibit viral entry; an optimal pH range for infection is 2–6 (Zimmer *et al.*, 2013). Following fusion, the RNP complex is released into the cytoplasm where viral mRNA is synthesized. Uncoating of the RNP structure occurs with 20 minutes of virus entry. Recently it has been suggested that a large portion of the M protein remains within the endosomal membrane and the remaining M protein migrates to the nuclear pore complex (Mire *et al.*, 2010).

The viral RdRP (L and P protein) transcribes a 47-nucleotide leader RNA that ends with an untranscribed AAA sequence on the 3' end (Whelan, 2008). The leader sequence is not modified with caps or polyadenylated tails, and it is used to negatively regulate host transcription at the nucleus (Kurilla *et al.*, 1982). Next, the RdRP transcribes the viral RNA genome into short mRNA fragments that encode the five viral proteins. Each viral gene is capped and polyadenylated poly(A) (Rodriguez L.L., 1999). The L protein recognizes a conserved sequence at the start of each individual mRNA and catalyzes a series of GDP-dependent enzymatic reactions that caps the 5' end (Ogino *et al.*, 2007) . A gene junction containing seven U-residues (U-stretch) on the 3' end of each gene causes the polymerase to stutter, creating the

corresponding poly(A) tail (Rodriguez L.L., 1999). At this point, synthesis of the upstream mRNA is terminated (Ball *et al.*, 1999; Wagner RR, 1996). Following the U-residues is a 10-nucleotide gene start sequence located at beginning of each gene which is essential for the RdRp to move on to the next mRNA region and re-initiate transcription (Barr *et al.*, 2002). By a mechanism that is not fully elucidated, the RdRp pauses at each intergenic region and re- initiation of transcription is not 100% efficient, therefore transcriptions of downstream genes produces 30% less mRNA for each subsequent gene as the RdRp progresses (Ball *et al.*, 1999). The polymerase complex transcribes each viral gene in a sequential and polar order; therefore, the N mRNA is produced in the largest quantity, followed by P, M, G, and L, respectively. (Rodriguez *et al.*, 1997).

Each viral gene is translated by host cell ribosomes. N, P, M, and L mRNAs are translated on free polyribosomes, and G mRNA on membrane-bound polyribosomes (Rodriguez L.L., 1999). The G protein contains a signal peptide that targets it to the endoplasmic reticulum (ER) for further processing before it is expressed on the cell membrane. Although host mRNA translation is inhibited upon virus infection, VSV mRNA translation still occurs (Lyles, 2007). It is not clear what mechanism allows the preferential translation of VSV mRNA over host mRNA. Recently it has been revealed that a cis-regulatory element located in the VSV mRNA is recognized by eukaryotic ribosome (also known as (a.k.a.) 80S ribosome) and enables selective translation of viral mRNA (Lee *et al.*, 2013). A role for the M protein has also been suggested for selective protein translation, but the exact mechanism is unclear (Mire *et al.*, 2011).

Genome translation is tightly coupled to RNA replication which results in the production of encapsulated full-length positive-sense RNA (anti-genome) (Whelan, 2008). Genome replication is dependent on N protein levels. When the N protein coats the viral anti-genome (encapsulation) the polymerase is able to transcribe the template; the N protein may be involved in modifying the polymerase so it bypasses the terminating junction region located at the 5' end of the upstream gene (Rodriguez

L.L., 1999; Whelan, 2008) The anti-genome is used as a template for production of negative-stranded RNA.

During viral assembly, the M protein connects with the RNP core and obstructs further viral transcription (Rodriguez L.L., 1999; Whelan, 2008). The M-RNP complex is also associated with cell surface trimeric G protein; this promotes the budding of packaged virion particles from the surface. VSV infection is very proficient with significant viral replication occurring before the host cell is killed; 10, 000 virus particles are produced from one infectious particle within an eight hour time frame in susceptible mammalian cells.

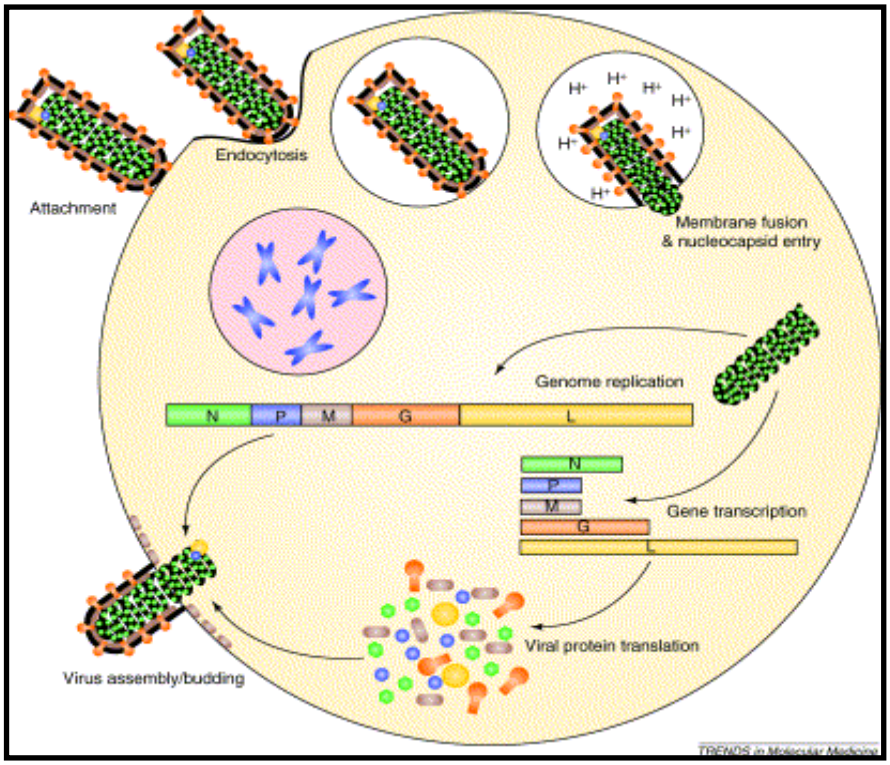

Figure 2: VSV Replication Cycle.

VSV glycoprotein enables the virus to attach to and fuse with host cells. Viral entry and fusion is facilitated by acidic endocytosis and viral particles enter the cellular endosomal trafficking pathway. The nucleocapsid containing the genomic RNA is released into the cytoplasm and viral replication begins. Viral polymerase transcribes the RNA genome into short mRNA fragments that encode the five viral proteins. After each viral gene is made, the polymerase starts transcribing full-length positive-sense RNA which is used as a template for the production of negative-stranded genomic RNA. Viral genes are translated into viral proteins using host cell ribosomes. Viral proteins and genomic RNA components are then assembled into complete virions and the virus buds through the host plasma membrane. (Reproduced with permission from (Lichty *et al.*, 2004).

1.1.2.4 Host–Viral Interactions Controlling VSV Pathogenesis

The IFN system is an important host defence against VSV replication and infectivity (Meraz *et al.*, 1996; Muller *et al.*, 1994). IFNs are cytokines released by host cells as a first line of defence against viral infection (Pestka *et al.*, 2004). There are three main subtypes of IFNs: Type 1 (IFNα/β), Type II (IFNγ), and Type III (IFNλ). In Type I IFN signaling, ligand binding to IFN receptors, IFN alpha/beta receptor (IFNAR)1, and IFNAR2 activates the Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (de Weerd *et al.*, 2012) (Figure 3). Tyrosine kinase 2 and JAK1 activate IFN receptors by phosphorylation and facilitate STAT1/2 recruitment. A number of IFN-stimulated genes (ISGs) implicated in the production of antiviral proteins are upregulated in response to IFN receptor binding (Wang *et al.*, 2012). In type II IFN signaling, IFNγ bind to IFN-gamma receptors (IFNGR)1/II(Platanias, 2005; Schroder *et al.*, 2004). Receptor binding leads to the autophosphorylation of JAK2 which then transphosphorylates JAK 1. Activated JAK 1/2 phosphorylate the IFNGRs creating docking sites for STAT1 proteins. Dimerized STAT1 proteins translocate to the nucleus and bind GAS (IFNγ-activated site) elements and initiate transcription of several antiviral ISGs. Both type I and II IFN signaling play a major role in VSV immunity (Chesler *et al.*, 2004; Chesler *et al.*, 2003; Meraz *et al.*, 1996; Moerdyk-Schauwecker *et al.*, 2013; Muller *et al.*, 1994). Mice deficient in type I IFN receptors or STAT-1 are extremely susceptible to VSV infection (Meraz *et al.*, 1996; Moerdyk-Schauwecker *et al.*, 2013; Muller *et al.*, 1994). Comparably, IFN-γ or IFNGR knockout mice are susceptible to VSV induced encephalitis (Komatsu *et al.*, 1997).

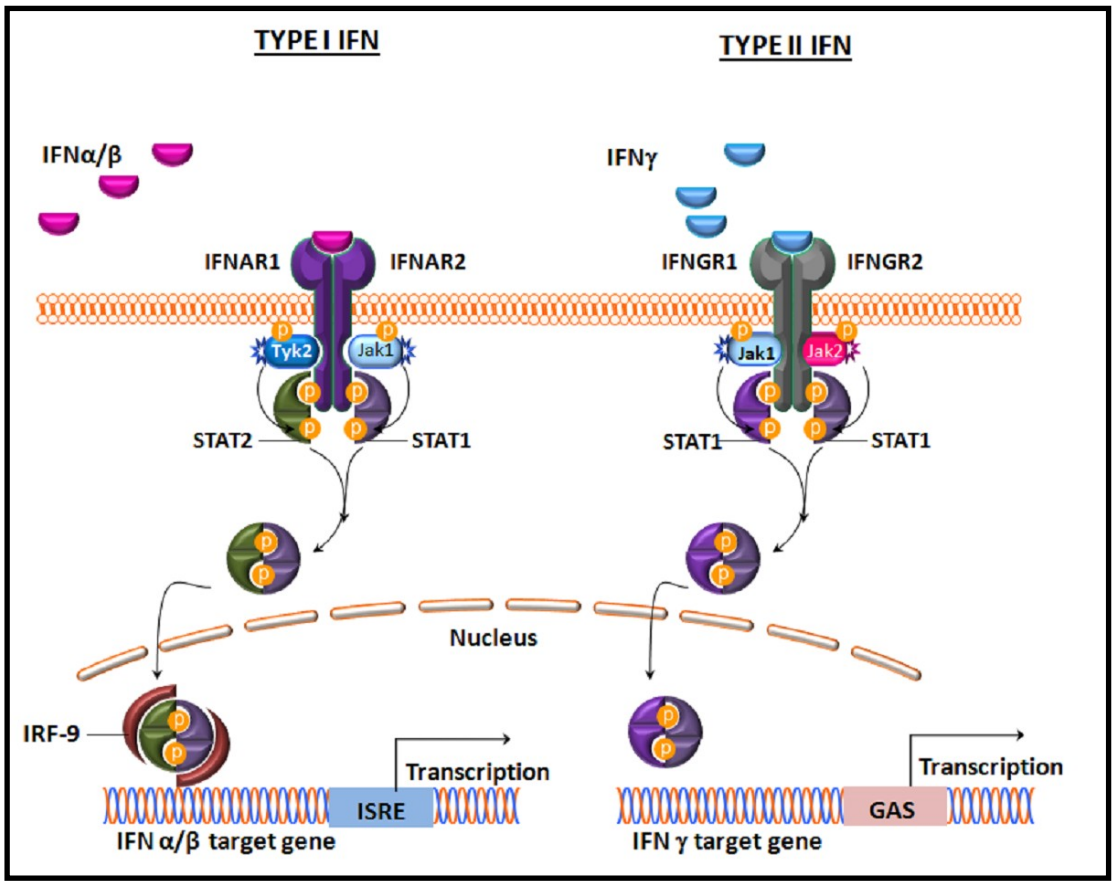

Figure 3: JAK/STAT signaling pathway activation by type I and type II IFNs

IFNα/β ligand binding to IFNAR1/IFNAR2 triggers receptor dimerization, which leads to the phosphorylation of associated JAKs (i.e. Tyk2 and JAK1) and the cytoplasmic tail of the receptors. The activated receptors facilitate STAT recruitment where it is phosphorylated by activated JAKs. STAT dimerizes, enters the nucleus, and binds to specific response elements in promoters to activate transcription of target genes. IFNγ ligand binds IFNGR1/IFNAR2 and stimulates JAK1/2 phosphorylation and STAT1 recruitment. Dimerized STAT-1 proteins translocate to, the nucleus and bind GAS elements.

IFN: Interferon, **Jak**: Janus Kinase, **STAT:** Signal Transducer and Activator of Transcription, **Tyk**: Tyrosine Kinase, **ISRE:** Interferon-Stimulated Response Element, **IFNAR:** Interferon-alpha/beta receptor, **IFNGR:** Interferon-gamma receptor, **GAS**: IFNγ--activated site, **IRF:** Interferon regulatory factor, **P**: Phosphorylation

VSV has devised methods to hamper its detection and elimination by the host. The majority of these evasion mechanisms are carried out by the M protein. Cytosolic VSV M protein attempts to shut down host antiviral and inflammatory responses by targeting STAT signaling (Terstegen *et al.*, 2001). The M protein of VSV blocks tyrosine phosphorylation of STAT-3 and interleukin (IL)-6–induced STAT-1 and STAT-3 activation (Terstegen *et al.*, 2001). It is also evident that M protein blocks type I IFN signaling by suppressing human IFN-β gene expression (Ahmed *et al.*, 2003; Ferran *et al.*, 1997; Stojdl *et al.*, 2003). Viruses expressing mutant forms of the M protein do not inhibit IFN-β induction. VSV inhibits IFN-β gene expression by inhibiting host transcription in the nucleus and by blocking host polymerase function. M protein inhibits all three host RNA polymerases (I-III), thereby repressing transcription from RNA polymerase–dependent promoters (Ahmed *et al.*, 1998) VSV M protein likely inhibits host polymerase through inactivation of a transcription initiation factor required by all three RNA polymerases (I-III). Transcription initiation factor, TFIID, of RNA polymerases II was identified as a target of VSV M protein (Lyles, 2000).

Upon viral infection, a portion of the M protein is targeted to the nuclear pore complex, where it bi-directionally restricts nucleocytoplasmic transport of RNA and obstructs host transcription (Black *et al.*, 1994; Black *et al.*, 1992; Black *et al.*, 1993; Faria *et al.*, 2005; Ferran *et al.*, 1997; Petersen *et al.*, 2000). Residues 51–59 of the M protein mediate the inhibition of host transcription. Unlike the wildtype (wt) M protein, a mutant version of the M protein expressing a methionine to arginine point mutation at residue 51 cannot reach the rim of the nuclear envelope. In order to block host transcription, M protein must bind host mRNA export factor Rae1/mrnp41 and nucleoporin Nup98 protein (Faria *et al.*, 2005; von Kobbe *et al.*, 2000). These complex formations are critical for the inhibition of host transcription and can block the nuclear export of IFN-β mRNA (Rajani *et al.*, 2012; Stojdl *et al.*, 2003). VSV M protein is not recruited to the nuclear envelope without Nup98 assistance (von Kobbe *et al.*, 2000). M protein is very potent and fast acting (Black *et al.*, 1992). Low

quantities of the protein strongly reduce transcription and the effects are visible within hours of infection.

1.1.3 VSV as an OV

VSV has emerged as a prototypical OV and possesses many features characteristic of an ideal OV. VSV exposure in the general population is extremely low; therefore, many people do not have any pre-exiting neutralizing antibodies to the virus that would limit its use as an oncolytic virotherapy (Lichty *et al.*, 2004; Roberts *et al.*, 1999). Moreover, VSV is generally non-pathogenic in humans; it is rapidly cleared by the immune system with little, if any, symptoms. VSV has broad tissue and cell tropism. The virus can successfully infect many cancer cell types in humans due to the ubiquitous specificity of the VSV-G protein. When tested on the 60 human cancerous cell lines included in the National Cancer Institute (NCI60) panel, VSV proved to be highly lytic in the majority of the cell lines. As a result of diminished or defective IFN signaling in cancer cells, VSV efficiently replicates in and kills malignant cells (Figure 4). Normal, healthy cells with intact IFN signal transduction elicit strong antiviral responses and impede viral infection. Because the M protein has a strong impact on virulence and pathogenicity (as described above), mutant forms of VSV, VSV-AV1, and VSV MΔ51 were designed to improve the safety profile of the virus (Stojdl *et al.*, 2003). VSV-AV1 possesses a mutation in methionine 51 (M51R), and the methionine 51 residue is completely deleted in VSV-MΔ51. Attenuated mutant VSV strains enhance IFN production in untransformed healthy cells and are thus very susceptible to the resulting IFN-induced antiviral response. Essentially, these mutants retain their ability to lyse IFN-deficient neoplastic cells. VSV-MΔ51 and VSV-AV1, unlike wt VSV, are incapable of blocking host transcription and nucleocytoplasmic RNA transport and, because of their improved safety profile, they are exclusively applied to oncolytic virotherapy (Petersen *et al.*, 2000; Stojdl *et al.*, 2003).

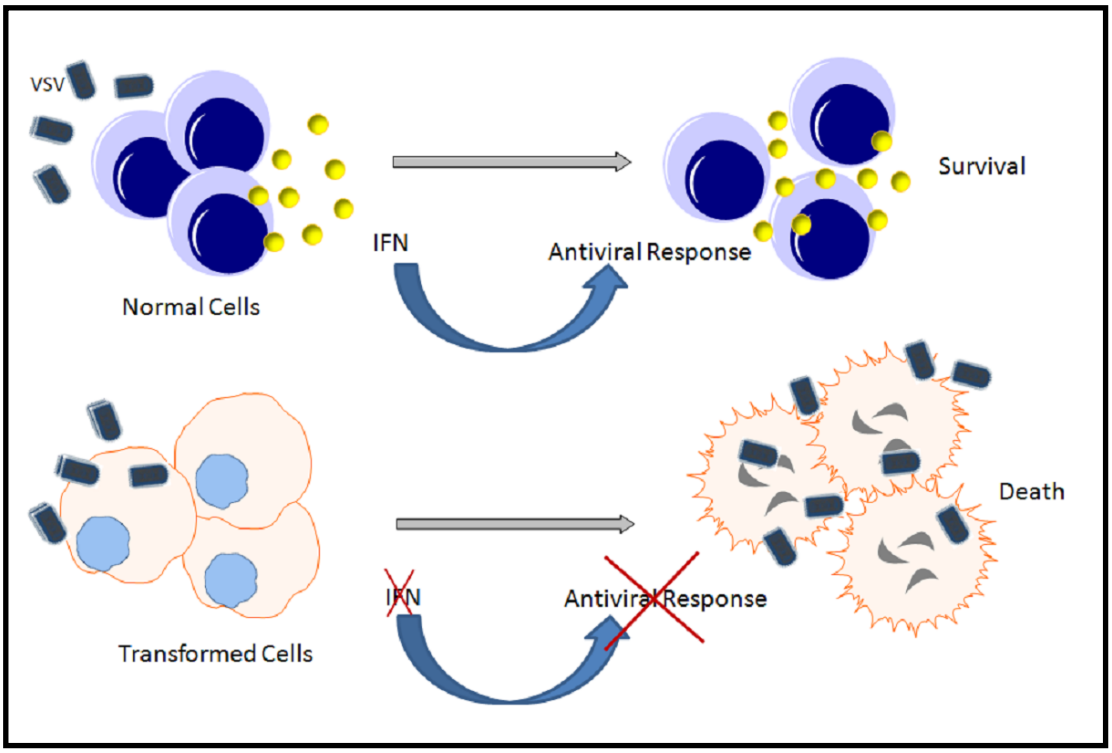

Figure 4: Paradigm of VSV Oncolysis

VSV preferentially replicates in and kills tumor cells while sparing normal cells. Regulated cell growth, and IFN-mediated antiviral responses, protect normal cells from VSV-induced lysis. Uncontrolled growth, and defects in IFN signaling, are exploited to enable viral oncolysis in cancer (transformed) cells.

VSV: Vesicular stomatitis virus, **IFN:** Interferon

1.1.4 Other OVs

1.1.4.1 Measles Virus (MV)

Measles virus (MV) is a negative-strand RNA virus and member of the *Paramyxovirus* family (Griffin, 2007). MV cell surface protein, hemagglutinin (HA), binds to cluster of differentiation (CD46) and signaling lymphocyte–activating molecule (SLAM) receptors on host cells (Dorig *et al.*, 1993; Naniche *et al.*, 1993; Tatsuo *et al.*, 2000). CD46 receptor is commonly overexpressed on cancer cells and mediates MV cytotoxicity (Fishelson *et al.*, 2003; Yanagi, 2001). Cell death is induced by the formation of syncytia, the fusion of neighboring cells; this augments the cytopathic effect of the virus (Anderson *et al.*, 2004; Galanis *et al.*, 2001). Normal cells do not express high levels of CD46, and their low-density receptor levels limit MV-mediated cytotoxicty (Anderson *et al.*, 2004). MV oncovirotherapy has been established in several cancer models including ovarian cancer, breast cancer, prostate cancer, and lymphomas (Grote *et al.*, 2001; Iankov *et al.*, 2010; Msaouel *et al.*, 2009; Peng *et al.*, 2006).

1.1.4.2 Adenovirus (Ad)

Adenovirus (Ad) is a DNA virus belonging to the A*denoviridae* family (Davison *et al.*, 2003). Ads have emerged as ideal OVs due to their ability to effectively lyse cancer cells and spread to surrounding cancer cells. A large group of Ads have been extensively modified and designed to specifically target cancer cells and to enhance the immune response. Many oncolytic Ads co-express therapeutic transgenes (i.e. Il-12, Il-18, relaxin, and decorin) that promote anti-tumor immune responses, T-lymphocyte and natural killer cell activation, viral spreading, and cancer cell lysis (Choi *et al.*, 2011; Choi *et al.*, 2010; Kim *et al.*, 2006; Lee *et al.*, 2006). Other Ads have modified genes and promoter regions that enhance tumor-

specific killing. Ads have been engineered to target p53-deficient cancers (ONYX-015), prostate-specific antigen (PSA)-expressing prostate cancers (CN706), telomerase-positive cancers (Ad-mTERT- Δ19), and hypoxic tumors (Ad-HRE(12)/hAFPΔ19 and HYPR-Ad) (Bischoff *et al.*, 1996; Khuri *et al.*, 2000; Kim *et al.*, 2003; Kwon *et al.*, 2010; Post *et al.*, 2003; Rodriguez *et al.*, 1997).

1.1.4.3 Reovirus

Reovirus (**r**espiratory **e**nteric **o**rphan virus) is a double-stranded RNA virus belonging to the *Reoviridae* family (Jackson *et al.*, 1973). Its name is an acronym of "the respiratory and enteric tract", two organs from which it is commonly isolated (Kelly *et al.*, 2009). Reovirus type 3, Dearing strain, has been extensively studied as an oncolytic virus (Stoeckel *et al.*, 2006; Yap *et al.*, 2008). More than 30% of cancers are defective or aberrant in Ras signaling, a pathway that affects many cellular functions, including cell proliferation, differentiation, and survival (Bos, 1989). Overactivation of Ras signaling facilitates reovirus replication in cancer cells (Norman *et al.*, 2000). Healthy cells with normal Ras signaling limit virus replication. Reovirus can effectively kill breast, colon, ovarian, melanoma, bladder, and pancreatic cancers (Errington, White*, et al.*, 2008; Etoh *et al.*, 2003; Hirasawa *et al.*, 2002; Kilani *et al.*, 2003; Norman *et al.*, 2002)}. Cancer cells lysed by reovirus release many cytokines that activate anti-cancer adaptive immunity (Errington, Steele*, et al.*, 2008; Errington, White*, et al.*, 2008). The anti-tumor effect can be amplified by antigen-presenting dendritic cells (DCs) that present tumor antigen to cytotoxic T cells (Prestwich *et al.*, 2008).

1.2 Chronic Lymphocytic Leukemia

Cancer is a pathological process that leads to the irregular and uncontrolled proliferation of cells and tissues (Cooper, 2000). In general, cancers are divided into two groups: 1) solid tumors, where abnormal swelling or growth of tissue creates localized masses of cancer cells or 2) hematological malignancies, where cancers form within the blood, bone marrow, or lymphatic system. Leukemia, lymphoma, and myeloma cancers are the three main types of hematologic cancers (Lichtman, 2008). Leukemia originates in the bone marrow and is a cancer of the blood (Cooper, 2000). Leukemias are further categorized in acute and chronic subtypes and disease progression is either rapid or slow, respectively. The most common type of leukemia in adults is Chronic lymphocytic leukemia (CLL) (Perez *et al.*, 2011; Shanshal *et al.*, 2012). CLL is the focus of this research; it is discussed in greater detail in the following sections.

1.2.1 Epidemiology

CLL is a B cell malignancy characterized by the accumulation of a monoclonal population of functionally defective lymphocytes (Garcia-Munoz *et al.*, 2012; Shanshal *et al.*, 2012). CLL is the most prevalent leukemia in Western countries, predominantly affecting older individuals. Accounting for ~30% of leukemias in the adult US population, there are approximately six cases per 100,000 persons diagnosed each year (Perez *et al.*, 2011; Shanshal *et al.*, 2012). The rate of incidence rises significantly in elderly (>65 years old) patients, increasing to ~13 cases per 100,000. The average age of clinical diagnosis is 70 years; although rare, <2% patients younger than 45 years old have also been diagnosed (Gribben, 2010; Hernandez *et al.*, 1995). The proportion of men to women diagnosed with CLL is 1.2–2:1 and this ratio remains relatively steady over various age groups (Perez *et al.*, 2011; Shanshal *et al.*, 2012). In 2011, there was an estimated 14,570 cases of CLL

reported in the United States of which, 58% were male and 42% were female (Siegel *et al.*, 2011). A 10-year relative survival rate (observed survival time of diseased patient compared to the expected survival without disease) of 65% and 55% was observed in CLL patients >70 years of age and between 70–79 years of age, respectively (Brenner *et al.*, 2008). In 2009, the median age of death was 79 years with an average of 1.6 per 100,000 patients dying every year from the disease (Gribben, 2010; Redaelli *et al.*, 2004). In recent years, survival rates have been found to be disproportionately higher in males than in females (Brenner *et al.*, 2008). The disease is more common among Caucasians from Australia, North America, and Europe compared to African Americans, Hispanics, Native Americans, or Asian populations (Gribben, 2010). Interestingly, CLL accounts for only 10% of all leukemias in Asian countries and migration studies showed that the CLL incidence rates in Asians living or born in the United States remained lower over time, suggesting a genetic versus environmental impact (Gale *et al.*, 2000; Haenszel *et al.*, 1968; Redaelli *et al.*, 2004; Shanshal *et al.*, 2012).

1.2.2 Etiology and Risk Factors

The causative element(s) for CLL is not currently known (Redaelli *et al.*, 2004; Shanshal *et al.*, 2012). CLL is a familial cancer, with studies showing an inherited susceptibility to the disease. The risk of developing CLL is increased by 2 to 7-fold in patients with a family history of the disease, a stark contrast to other indolent hematological malignancies, such as Non-Hodgkin lymphoma, where risk increases by only 1.8-fold (Goldin *et al.*, 2009; Shanshal *et al.*, 2012). Familial CLL is very aggressive, presenting ~10 years earlier than in sporadic cases (in which no family member has had the disease); however, it has been debated that increased screening in families may be the underlining reason for an earlier diagnosis (Houlston *et al.*, 2008; Houlston *et al.*, 2003; Shanshal *et al.*, 2012). Females from familial cases have an increased incidence rate, possibly as a result of high genetic predisposition and genetic penetrance (Crowther-Swanepoel *et al.*, 2010). The underlying genetic

element(s) for familial association of CLL has not been identified, but human leukocyte antigen (HLA) haplotype and immunoglobulin heavy-chain variable region (IgVH) gene mutations have been ruled out as potential causes (Houlston *et al.*, 2008).

The development of CLL has not been linked to environmental risk factors, and a causal relationship between CLL pathogenesis and known carcinogens (i.e. ionizing radiations, pesticides, sunlight, alkylating agents) has not been documented (Hamblin *et al.*, 1999; Redaelli *et al.*, 2004; Shanshal *et al.*, 2012). Hepatitis C, Epstein-Barr virus, and Human T-cell lymphotrophic virus (HTLV)-I and -II are linked to a number of cancers and tumorigenesis, but have not been correlated to CLL development.

1.2.3 Clinical Diagnosis, Symptoms and Signs

Since CLL is an indolent and chronic disease, one quarter of patients do not exhibit any symptoms at all, but those who do present a wide range of symptoms and signs, including fatigue, swollen lymph nodes and elevated lymphocyte counts (Redaelli *et al.*, 2004; Shanshal *et al.*, 2012). Other symptoms associated with CLL include up to a 10% reduction in body weight (within a six-month period); persistent (>two weeks) fevers and night sweats that result without infection; bleeding; and occasionally, acquired immunodeficiency disorders and autoimmune complications. Several organs become enlarged during disease progression; lymphadenopathy is the most common and earliest detected, followed by splenomegaly and hepatomegaly, the least common. The Waldeyer's ring, located in the pharynx, can also increase in size. CLL cells can also infiltrate non-lymphoid organs, specifically the skin, causing leukemia cutis resulting in facial lesions, blisters, and ulcers. Membranoproliferative glomerulonephritis, a kidney disorder, has also been observed in some cases.

CLL is diagnosed when white blood cell levels elevate above 5000

B-lymphocytes/μL (lymphocytosis) for three months or longer (Shanshal *et al.*, 2012). Diagnosis is made by peripheral blood smear examination, and the maturity state of the B cells and their small size; the latter two are assessed by flow cytometry. Flow cytometry testing is also used to determine the expression of CD19, CD20, CD23, and CD5 immunotypes, all together representing clonal B cells. CD19 antigen, a cell surface phosphoglycoprotein, and CD20, an activated-glycosylated phosphoprotein, are expressed on all B-cell lineages with the exception of CD19, which is not present on plasma cells (Kozmik *et al.*, 1992). CD23, a low affinity immunoglobulin (Ig) E receptor, is expressed on mature B cells, antigen presenting cells, and platelets (Cramer *et al.*, 2011). The soluble form of CD23 is associated with an aggressive CLL clinical course leading to bone marrow failure, tumor growth, and reduced survival (Knauf *et al.*, 1997; Molica *et al.*, 1999; Saka *et al.*, 2006). The CD5 glycoprotein, generally a marker for normal T cells, is highly expressed in CLL (Dalloul, 2009). The effects of CD5 expression have been documented in other cancers and the protein is known to protect against autoimmunity and inhibits anti-tumoral immune responses. Hypogammaglobulinemia, a deficiency of Ig production, is observed in a few patients, primarily affecting IgA, IgM, and IgG levels, and thus increases the chance of pathogen infection (Gentile *et al.*, 2009; Pangalis *et al.*, 2002). Beta(β)2-microglobulin (a component of the Major Histocompatibility Complex class I molecule) levels are increased in the serum of CLL patients, and is believed to have a strong predictive value for treatement (Gentile *et al.*, 2009). CLL cells, which originate in the bone marrow, can cause bone marrow failure in some patients. When more than 30% of monoclonal lymphocytes infiltrate the bone marrow, a patient is deemed at risk. Progressive marrow failure may result in anaemia or thrombocytopenia via the production of erythrocyte- and platelet-specific antibodies, respectively (Redaelli *et al.*, 2004).

1.2.4 Clinical staging of CLL

There are two different systems for the clinical staging of CLL: the Rai staging system, defined by Dr. Kanti Rai, which is used more often in the United States, and the European Binet classification system, created by Dr. Jacques-Louis Binet(Binet *et al.*, 1981; Rai *et al.*, 2004). Both systems use clinical findings (i.e. physical examination and blood work to predict prognosis and survival).

The original Rai system, established in 1975, was comprised of five stages (0–IV), based on lymphocyte blood count, lymph node count, and spleen and liver enlargement (Rai *et al.*, 2004). In 1985, the classification was modified to a three-stage system based on risk level: low (previously stage 0), intermediate (previously Stages I and II) and high (previously Stages III and IV) due to similar survival rates for patients in each stage (Table 1). Life expectancy is highest in the low-risk category. This group is defined by blood lymphocytosis >5000/μL and bone marrow lymphocytosis >40%. Approximately 30% of patient fall into the low-risk category (Rai *et al.*, 2004; Redaelli *et al.*, 2004; Shanshal *et al.*, 2012). Patients in the intermediate-risk group have a median life expectancy of eight years. Lymphocytosis is similar to that observed in low risk patients; however, nodes, spleen, and/or liver are enlarged. The-high risk group has the lowest life expectancy with two years; patients exhibit lymphocytosis, anaemia, and/or thrombocytopenia with or without the presence of enlarged nodes, spleen, and/or liver.

RAI STAGING:

Stage	Modified Rai Category	Features	Median Survival (yr)
0	Low risk	Lymphocytosis	13+
I	Intermediate risk	Lymphocytosis and enlarged nodes	8
II		Lymphocytosis and enlarged spleen or liver	
III	High risk	Lymphocytosis and anaemia	2
IV		Lymphocytosis and thrombocytopenia	

BINET STAGING:

Stage	Features	Median survival (yr)
A	Haemoglobin ≥10 g/dL, platelets ≥100,000/mm3, and <3 enlarged areas	15
B	Haemoglobin ≥10 g/dL, platelets ≥100,000/mm^3, and ≥3 enlarged areas	5—7
C	Haemoglobin <10 g/dL, platelets <100,000/mm^3, and any number of enlarged areas	3

Table 1: CLL Clinical Staging Systems.

Adapted with permission from (Rai et al., 2004).

The Binet staging system, developed in 1981, defines three clinical stages (A–C) based on haemoglobin and platelet levels, and the number of enlarged lymphoid sites (Binet *et al.*, 1981). In all three stages, patients have blood and bone marrow lymphocytosis (Rai *et al.*, 2004). In stage A, haemoglobin and platelet counts are less than 10 g/dL and 100,000/mm^3 respectively, and there are fewer than three sites of palpably enlarged lymphoid areas (Rai *et al.*, 2004; Redaelli *et al.*, 2004; Shanshal *et al.*, 2012) .The median survival time for stage A patients is 15 years. Symptoms in stage B patients are the same as stage A, with the exception of patients having three or more palpably enlarged lymphoid areas; survival decreases to five to seven years. Stage C patients are anaemic (haemaglobin < 10g/dL) or thrombocytopenic (<100,000/mm^3) and have any number of enlarged lymphoid sites; the median survival for patients in this category is three years.

The Rai and Binet classification systems are used worldwide, with Rai predominantly being used in North America and Binet in the remaining parts of the world. It has been suggested that these two staging systems be combined, however, this has yet to occur in the clinic.

1.2.5 Prognostic Factors in CLL

Numerous prognostic factors have been identified for CLL treatment, response and, survival. The best characterized and most significant prognostic markers are CD38, Zeta-associated protein 70 (ZAP-70), immunoglobulin heavy chain variable region (IgVH) mutational status, and chromosomal abnormalities.

1.2.5.1 CD38 Expression

CD38, also known as cyclic ADP ribose hydrolase, was first identified as a marker of activation and proliferation in T lymphocytes (Funaro *et al.*, 1990).

Human CD38 is a 45 kDa single-chain type II transmembrane glycoprotein (Malavasi *et al.*, 2008). Functionally, CD38 molecule is a dimeric receptor and enzyme; the catalytic site is located at the interface between the two monomers (Funaro *et al.*, 1990; Munshi *et al.*, 2000). In humans, CD38 is also expressed on the surface of both mature and immature B lymphocytes, including B cell precursors, germinal center B cells, and plasma cells (Malavasi *et al.*, 2011). Circulating B cells and memory B cells have low CD38 levels. CD38 can localize to the cytoplasm and/or the nucleus of various cells; however, in human B lymphocytes, the molecule is expressed on the cell surface (Malavasi *et al.*, 2011; Malavasi *et al.*, 2008). Enzymatically active CD38 participates in Ca^{2+} signaling, cell adhesion, and intracellular signal transduction.

High CD38 expression is associated with poor prognosis and more aggressive disease. Patients with elevated CD38 receptor expression have higher rates of lympadenoptahy and hepatomegaly. Moreover, the time span from initial symptoms to treatment, the response to therapy and the survival rate decrease drastically with receptor presence (Chevallier *et al.*, 2002; Cramer *et al.*, 2011; Hayat *et al.*, 2006; Jelinek *et al.*, 2001). Cell surface expression >30% is generally accepted as the threshold for CD38 positivity and significance. Accumulation of CD38 receptor on CLL cells was originally believed to be a surrogate marker for IgVH mutation status, another CLL prognosis factor; however, gene analysis in patients showed that approximately 28% of patients refute this association (Hamblin *et al.*, 2002). CD38 expression is transient and variable throughout disease progression therefore making it difficult to association with IgVH mutational status (Hamblin *et al.*, 2000; Hamblin *et al.*, 2002). Using CD38 as a candidate marker to determine the proper time to initiate treatment has its limitations due to the fluctuating nature of receptor levels during disease progression (Malavasi *et al.*, 2011). Nonetheless, CD38 remains an independently reliable prognostic indicator that is easily detected by flow cytometry (Rai *et al.*, 2004). CD38 monoclonal antibody (mAb) therapy is being tested clinically and has been proposed as an alternate therapeutic for CLL (Malavasi *et al.*, 2011; van der Veer *et al.*, 2011). In humans, CD38 is also present on non-

lymphocytic cells and is ubiquitously expressed in almost all tissues including the brain and retina, thus raising the possibility of safety limitations (Malavasi *et al.*, 2011).

1.2.5.2 Zeta-chain–Associated Protein-70 Expression (ZAP-70)

ZAP-70 is a 70 kDa cytoplasmic tyrosine kinase that associates the zeta subunit of the T-cell receptor (TCR) and natural killer cells (NK) (Wang *et al.*, 2010). It is a member of the spleen tyrosine kinase (Syk) family and is expressed on normal T cells and NK cells, and has a critical role in initiating TCR signaling (Hamblin *et al.*, 2005; Wang *et al.*, 2010). ZAP-70 deficiency has been linked to defective TCR signaling and severe combined immunodeficiency in mice and humans, revealing a vital role for the protein in homeostasis (Chan *et al.*, 1994; Elder *et al.*, 1994). Although not normally expressed on healthy B lymphocytes, ZAP-70 is present on CLL B cells as an adaptor protein, boosting B cell receptor (BCR) signaling, or as a negative regulator of Syk, a related B cell protein–tyrosine kinase (Chen *et al.*, 2008; Chen *et al.*, 2002; Gobessi *et al.*, 2007). Enhanced BCR signaling by ZAP-70 has been implicated in aggressive disease progression in CLL patients (Chen *et al.*, 2002). As with CD38, ZAP-70 expression in leukemic cells was believed to correlate with IgVH mutational status; however, this has been disputed and ZAP-70 protein is considered an independent prognostic marker (Krober *et al.*, 2006).

ZAP-70–mediated BCR signaling appears to be dependent on the presence of heat shock protein 90 (Hsp90), a chaperone protein found in many cancers (Castro *et al.*, 2005). Hsp90 binds substrate (client) proteins to assist in protein stabilization and translocation. In CLL, Hsp90 binds and stabilizes ZAP-70, preventing cell death, an interaction not found in T cells. Based on these findings, Hsp90 inhibitors have emerged as possible therapies for CLL. ZAP-70 is often analyzed by flow cytometry concomitantly with CD38 analysis. Prognosis can be grouped into three categories

based on CD38 and ZAP-70 levels. Patients positive for both markers have the worst prognosis, patients with at least one marker have an intermediate risk level, and CD38-negative and ZAP-70 negative patients having the best prognosis (Cramer *et al.*, 2011).

1.2.5.3 Mutational Status of Immunoglobulin Heavy Chain Variable Region (IgVH) Genes

BCRs are integral membrane-bound antibodies (Abs) or Igs located on the surface of B-lymphocytes (Janeway CA Jr, 2001; Treanor, 2012). These receptors are secreted as antibodies in the bloodstream upon B-cell activation. BCRs are tetramers containing two identical heavy chains and two identical light chains. In humans, there are five types of Igs that are defined based on their heavy chain region: IgA, IgD, IgE, IgG, and IgM. In their mature state, B cells can express two isotypes: IgM and IgD (Treanor, 2012). During B-cell maturation, somatic hypermutation (SHM), a mechanism by which point mutations are introduced into the variable region of the Ig gene (IgVH), generates immune diversity thus increasing antibody affinity to various invading pathogens. IgVH mutational status has significant prognosis implications in CLL. Patients with unmutated IgVH, thus displaying greater than 98% sequence homology with germ-line IgVH, have advance and progressive disease with a median survival of ~8 years (Hamblin *et al.*, 1999; Shanshal *et al.*, 2012). Survival rate increases to 24 years in patients with SHM in the IgVH region (<98% homology) and a more benign clinical course is observed. Fifty-five percent of CLL cases display mutated IgVH, while the remaining 45% are not mutated (Hamblin *et al.*, 1999). Analysis of CLL patient genes demonstrated that unmutated IgVH genes in CLL is a significant and independent prognostic indicator (Oscier *et al.*, 2002).

1.2.5.4 Chromosomal Abnormalities

Cytogenetics testing in CLL has identified subsets of patients with distinct disease pathogenesis and prognosis (Gozzetti *et al.*, 2004). Fluorescence in Situ Hybridization (FISH) analysis is routinely used to test for chromosomal abnormalities in CLL samples due to its ability to sequence DNA in non-dividing cells. CLL cells are arrested in the G_0/G_1 phase of the cell cycle and do not divide; therefore, this testing method has proved to be ideal. FISH is a cytogenetic technique used to detect whether specific DNA sequences are present or absent on a chromosome (Tsuchiya, 2011). After this technique was introduced for the analysis of CLL abnormalities, 80% of cases were found to have at least one chromosomal aberration (Dohner *et al.*, 2000). Five major classes of chromosomal abnormalities were identified in 325 cases analyzed. Deletion in chromosome 13 (del[13q]) was most frequently observed, with 55% of patients possessing the deletion. Deletion of chromosome 11 (del[11q]), trisomy in chromosome 12 (trisomy 12q), deletion of chromosome 17 (del[17p]), and deletion of chromosome 6 (del[17q]) were the next most common abnormalities occurring in 18%, 16%, 7%, and 6% of cases, respectively. Additional, but less frequent chromosomal abnormalities were also detected including translocation 14q32, aberrations of 6p, 2q, 12p, and trisomy in chromosomes 3 and 8 (trisomy 3q, trisomy 8q) (Odero *et al.*, 2001). The majority of the 325 cases revealed only one aberration; however, a few cases displayed multiple abnormalities (Dohner *et al.*, 2000).

Median overall survival is significantly longer in patients with del[13q]; patients typically survive 133 months (Dohner *et al.*, 2000). The median survival is 114 months for patients with normal karyotype, 11 months for trisomy 12 patients, 79 months for del[11q] patients, and 32 months for del[17p] patients. Weight loss, rapid disease progression, poor survival, low haemoglobin and platelet levels, abdominal extension, and lymphadenopathy are common in patients with del[11q] and del[17p] deletions (Dohner *et al.*, 2000; Dohner *et al.*, 1997). In del[11q] patients, survival is age-dependent; young patients (<55 years) have a very poor

survival rate. Two genes are affected by the del[11q] and del[17p] deletions: the ataxia teleangiectasia mutated (ATM) and the tumor protein 53 (TP53) gene, respectively (Stilgenbauer *et al.*, 1996; Zenz *et al.*, 2008). ATM is activated by DNA damage which regulates apoptotic cell death via tumor suppressor protein p53 (Chung *et al.*, 2012). The deletion of these genes may explain the chemoresistance observed in these subsets of patients as chemotherapy induces cell death through the p53 DNA damage signaling pathway and del[11q] and del[17p] patients cannot respond to genotoxic chemotherapies that act through this pathway (Rosenwald *et al.*, 2004). Collectively, these observations confirm the importance of FISH analysis in the prognosis and treatment of CLL.

1.2.6 CLL Treatment

CLL patients are treated based on their Rai and Binet staging. There is a standard wait-and-watch approach taken for individuals who fall under the Binet Stages A and B and Rai Stages 0, I, and II classifications because they do not display any apparent clinical symptoms (Shanshal *et al.*, 2012). This strategy is generally used in patients with early stage disease, as there is no evidence that early treatment will improve overall survival or prognosis. Therapy should commence as soon as patients become symptomatic (i.e. fever, fatigue, bone marrow failure, anaemia, thrombocytopenia or lymphadenopathy, etc) or display signs of aggressive disease progression (increased lymphocyte doubling).

Single-agent therapies are frequently used as first-line treatments. The purine analogue fludarabine has been reported to be the most effective drug for CLL treatment, although it is not recommend for use in elderly patients (Hamblin *et al.*, 2002). Common side effects following treatment include myelosupression (bone marrow suppression), increased risk of infection, anaemia and T-cell dysfunction (Boogaerts *et al.*, 2001). There is an overall response rate of ~70% but complete response rate is lower, varying from 20%–63%. Chlorambucil, an alkylating agent, is

also used as first line therapeutic. It has a complete response rate of 50–60% but can also cause myelosupression; seizures; hepatotoxicity, and in a few cases, secondary cancer (Andritsos *et al.*, 2002; Hamblin *et al.*, 2002). Fludarabine is used over chlorambucil in the United States and chlorambucil is more commonly used in Europe (Shanshal *et al.*, 2012). Another alkylating agent, bendamustine, was recently approved for use in the United States in patients who have not received any prior treatment (Knauf *et al.*, 2009). Bendamustine has displayed a better overall response rate in comparison to chlorambucil. Monoclonal antibody therapies ofatumumab (anti-CD20), alemtuzumab (anti-CD52), and rituximab (anti-CD20) are also used as first-line therapies (Robak, 2012; Shanshal *et al.*, 2012). In contrast to rituximab, the first monoclonal antibody approved for lymphomas, alemtuzumab, is able to target p53 mutated cells, and is therefore a better option for patients with del[11q] and del[17p] cytogenetic abnormalities (Shanshal *et al.*, 2012). Nonetheless, alemtuzumab increases the risk for opportunistic reactivation of cytomegalovirus and pneumocystis infections. Adverse events caused by rituximab treatment in CLL are associated with neutropenia and hypogammaglobulinaemia of IgG (Robak, 2012). Ofatumumab was recently approved as a single agent for the treatment of patients that are refractory to fludarabine and alemtuzumab therapy (Lemery *et al.*, 2010). Lumiliximab (anti-CD23) antibody is currently being investigated in clinical trials for the treatment of CLL (Byrd *et al.*, 2010).

Several combination chemotherapy regimens are used for CLL: fludarabine, cyclophosphamide, and rituximab (FCR) — the most frequently administered; pentostatin, cyclophosphamide, and rituximab (PCR), fludarabine, cyclophosphamide, and mitoxantrone (FCM); cyclophosphamide, vincristine, and prednisone (CVP); and cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) (Shanshal *et al.*, 2012). The application of each combination therapy depends on various factors, including age and inability to respond to other treatments (Andritsos *et al.*, 2002; Shanshal *et al.*, 2012).

Heamatopoietic stem cell transplantation is a last resort therapy for CLL patients (Gladstone *et al.*, 2012; Shanshal *et al.*, 2012). Stem cell transplantation is reserved for patients who are chemo-resistant and antibody resistant. It is rarely used due to the fact that most patients are elderly and are therefore not prime candidates for the aggressive treatment, which has many adverse effects. Moreover, the overall survival rate does not increase following transplantation.

1.3 Apoptosis

It is necessary for cells to maintain homeostasis in order to function properly (DeLong, 1998). Appropriate regulation and control of cellular homeostasis keeps cells in a steady state and balances cell survival and cell death. Excessive or malfunctional proteins are degraded or recycled in order to preserve cell integrity. However, various intracellular and extracellular factors —for instance stress, hypoxia, and invading pathogens — induce cellular signaling that shifts the balance toward cell death (Fulda *et al.*, 2010). Depending on the signal received by the cell, different forms of cell destruction can occur, including apoptosis, autophagic cell death, necrosis, osmotic lysis, necroptosis, and many others.

Apoptosis is one of the best characterized cell death mechanisms. The discovery of apoptosis can be dated back to the mid-1800s. In 1842, Carl Vogt described the principle of "natural cell death" when investigating toad embryo development (Cotter, 2009). From this discovery onward, the field of cell death has expanded rapidly. The name is a Greek term for the "falling/dropping off" of leaves from a tree (Kerr *et al.*, 1972). Apoptosis is a major form of active cellular destruction often referred to as type I programmed cell death (PCD). Apoptosis plays a pivotal role in normal physiological development, tissue integrity, homeostasis, and defence against human disease (Cotter, 2009; Danial *et al.*, 2004; Long *et al.*, 2012). Therefore, it is no surprise that dysregulation of apoptosis can lead to the development of a variety of pathological conditions including cancer, autoimmunity, embryonal death and neurodegenerative disease (Elmore, 2007; Lavrik *et al.*, 2005). Apoptosis induction is triggered by various internal and external signals, including heat shock, radiation, osmotic stress, cytotoxic drugs, infection, and oncogenic transformation.

Walther Fleming first described the morphological modifications associated with apoptosis in 1885 (Cotter, 2009). Apoptosis is a highly regulated cell suicide pathway, characterized by distinct morphological characteristics and biochemical

properties that differentiate it from other cell death categories. Morphological changes occur in both the nucleus and cytoplasm of apoptotic cells (Kerr *et al.*, 1972; Kroemer *et al.*, 2005). In the early stages of apoptosis, cells undergo chromatin condensation, DNA fragmentation, nuclear pyknosis, nuclear fragmentation, and cell shrinkage. In later stages, blebs (cell surface bubbles) and apoptotic bodies (fragments of dead or dying cells) are formed. In vivo, however, phagocytes engulf dying cells prior to the formation of apoptotic bodies. In stark contrast to death induced by necrosis, the plasma membrane remains intact throughout the entire apoptotic process.

Apoptosis elicits several energy-dependent biochemical processes that are specific to the cell death pathway. During apoptosis, there is 1) activation of proteolytic enzymes (caspases), 2) DNA and protein breakdown, and 3) cell membrane alteration (Kumar V, 2010). Caspases are **c**ysteine **aspa**rtic acid prote**ases** that are responsible for the execution of the apoptotic program. Caspases cleave thousands of protein substrates during the induction of apoptosis and target components of the cytoskeleton and nuclear scaffold, thus weakening cell integrity (Lavrik *et al.*, 2005). Apoptotic signaling activates initiator caspases (caspase-2, -8, -9, -10), which cleave and activate downstream effector caspases (caspase-3, -6, -7), creating the characteristic morphological and biochemical apoptotic features described above. Caspase-independent forms of cell death have also been observed and extensively studied. DNAase enzymes, which catalyze hydrolyses of the DNA, are activated by caspases and breakdown DNA within the cell. During apoptosis, the membrane lipid PS is flipped from the inner leaflet of the plasma membrane to the outer leaflet. When PS relocates to the extracellular surface, it functions as recognition marker for phagocytic cells (i.e. macrophages) which engulf and remove apoptotic cells before lysis, as not to damage neighboring cells.

Two central pathways mediate apoptosis: 1) intrinsic mitochondrial pathway and 2) extrinsic death receptor pathway (Elmore, 2007) (Figure 5). Although activated by different stimuli, the two distinct pathways ultimately converge to

activate downstream effector caspases. Depending on the cell type involved, the two routes can occur exclusively of each other (Sayers, 2011).

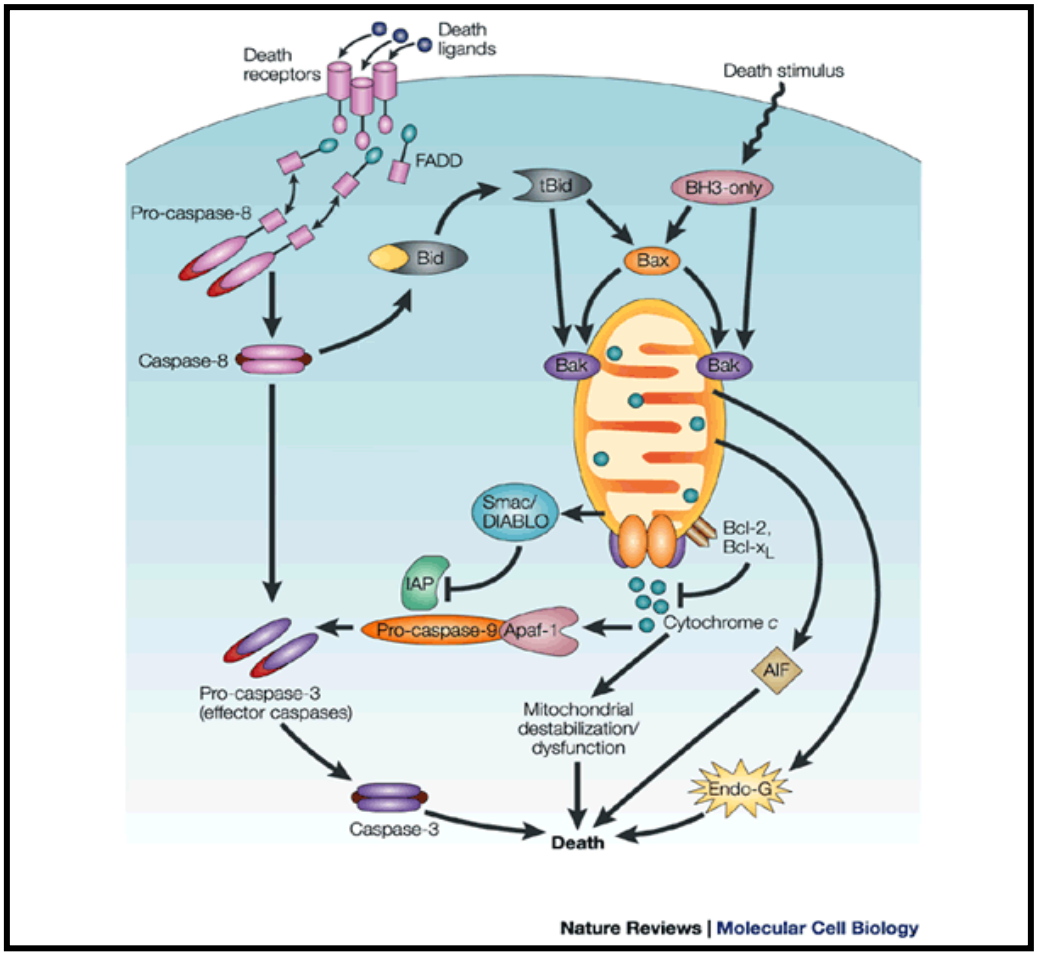

Figure 5: Intrinsic and Extrinsic Apoptosis.

Apoptosis can be activated by the extrinsic pathway or the intrinsic pathway**.** Extrinsic apoptosis is mediated by cell surface death receptors that act through initiator caspase-8, and intrinsic apoptosis is activated through intracellular signals which act through initiator caspase-9. Both pathways ultimately converge to activate effector caspase-3. The Bcl-2 family regulates intrinsic apoptosis through the inhibition (i.e. Bcl-2 and Bcl-x_L) or stimulation (i.e. Bax and Bid) of cytochrome c release. The IAP family also regulates intrinsic apoptosis by inhibiting downstream effector caspases. (Modified with permission from (Tilly, 2001)).

AIF: Apoptosis-Inducing Factor**, IAP:** Inhibitors of Apoptosis, **FADD:** Fas-associated death domain, **SMAC**: Second mitochondrial activator of caspases, **APAF-1:** Apoptotic protease activating factor-1

1.3.1 Intrinsic Apoptosis

The intrinsic mitochondrial pathway, as the name implies, is activated through intracellular stimuli (Elmore, 2007). Irreversible DNA damage, hypoxia, excessive cytosolic Ca^{2+} concentrations, oxidative stress, and viral infection can all stimulate intrinsic apoptosis signaling. The pathway is regulated by members of the B-cell lymphoma (Bcl-2) family of proteins which are subdivided into three groups based on their roles and structures:anti-apoptotic proteins, pro-apoptotic proteins, and BH3-only proteins (Cory *et al.*, 2002). Collectively, the Bcl-2 family governs the permeabilization of the outer mitochondrial membrane (OMM), which is an essential control point for apoptosis (Figure 5).

1.3.1.1 Bcl-2 Family

Bcl-2 family comprises a large number of structurally related proteins (Cory *et al.*, 2002; Youle *et al.*, 2008) (Figure 6). The family is categorized into three groups based on function and structure. Members are either pro-apoptotic (pro-death) or anti-apoptotic (pro-survival), therefore inducing or antagonizing the signaling pathway, respectively. The subfamilies are characterized by the number of Bcl-2 homology (BH) domains contained within their structure. Bcl-2 proteins contain one or more of the four BH domains (BH1-4), which facilitate heterodimeric interactions between the family members (Danial *et al.*, 2004). The anti-apoptotic subfamily contains Bcl-2, Myeloid cell leukemia factor-1 (Mcl-1), Bcl-extra long (Bcl-xL), Bcl-w, BCL2-related protein A1(A1/Bfl-1), and Bcl-2 homolog of ovary (Boo) (Igney *et al.*, 2002). Members of this group contain all four BH domains (BH1–4) with the exception of Mcl-1 that only contains the first three domains (BH1–3). Pro-death proteins are divided into two subgroups, the BH3-only subfamily and the pro-apoptotic subfamily. The BH3-only group, as the name denotes, only possesses one domain: BH3 (Happo *et al.*, 2012). The group is comprised of several members:

BH3 interacting domain death agonist (Bid); Bcl-2 interacting mediator of cell death (Bim); Bcl-2 antagonist of cell death (Bad); Bcl-2 interacting killer (Bik); Bcl-2 modifying factor (Bmf); phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP), commonly known as Noxa (meaning damage); p53 upregulated modulator of apoptosis (Puma); Blk; and hara-kiri (Hrk). The pro-apoptotic subgroup includes Bcl-2-associated X protein (Bax), BCL-2 antagonist or killer (Bak), and Bcl-2 related ovarian killer (Box)/Mtd. Bax, Bak, and Box contain multiple BH domains linked to a transmembrane (TM) domain.

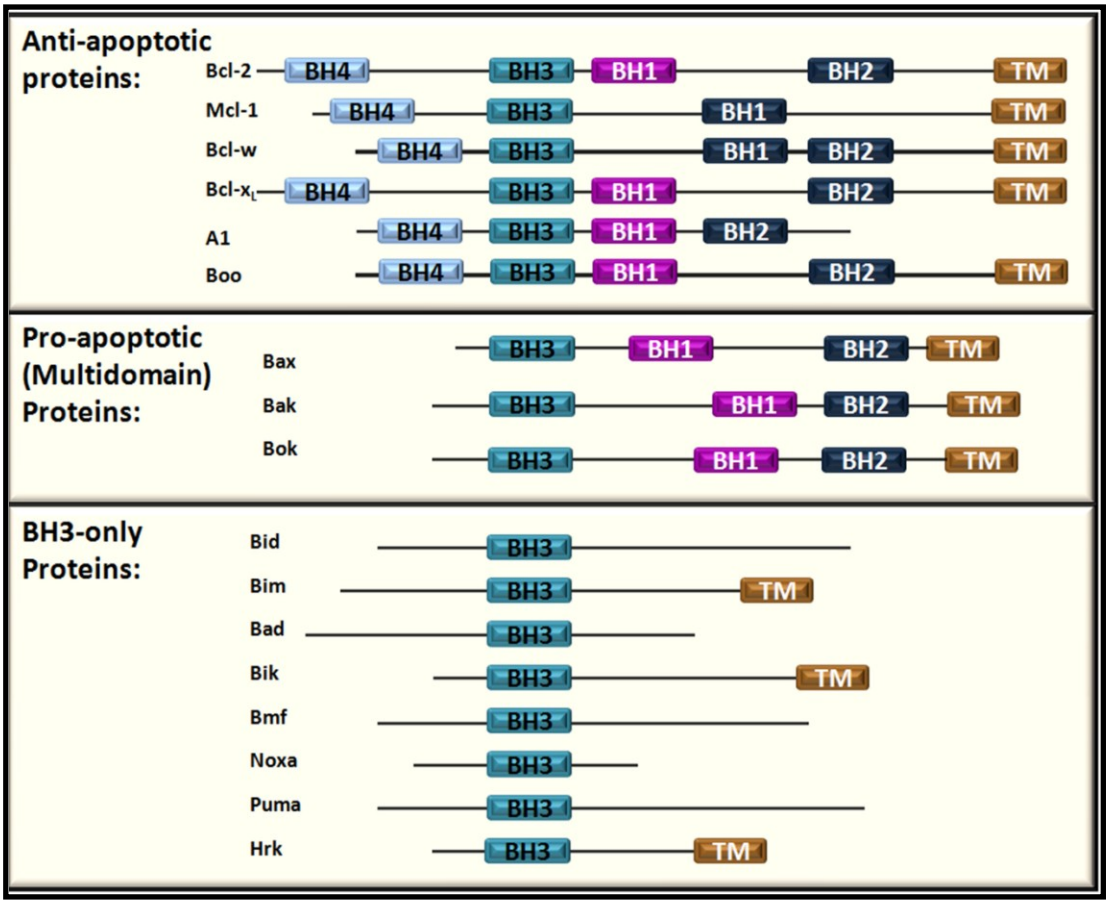

Figure 6: Bcl-2 family.

The Bcl-2 family comprises three subfamilies: 1) Anti-apoptotic, 2) Pro-apoptotic, and 3) BH3 only. The Bcl-2 family of proteins are classified based on their structural domain and function.

BH: Bcl-2 homology domain, **TM**: Transmembrane domain

(Modified with permission from (Strasser, 2005)

1.3.1.1.1 Anti-apoptotic Subfamily

Anti-apoptotic proteins contain three to four BH domains and a TM domain in their C-terminus. The BH1-3 domains form a hydrophobic binding pocket, referred to as the "BH grooves" that facilitate interaction with other Bcl-2 family proteins (Huang *et al.*, 2002). The C-terminus TM domain targets anti-apoptotic proteins to the cytoplasmic interface of the OMM, the nuclear membrane and/or the ER membrane (Borner, 2003). Certain members also localize to the cytoplasm. At these various subcellular locations, anti-apoptotic proteins bind and inhibit pro-apoptotic effector proteins. Bcl-2 is an integral membrane protein and, upon apoptotic induction, can relocalize from the nuclear envelope and ER membrane to the OMM (Akao *et al.*, 1994; Hockenbery *et al.*, 1990). Anti-apoptotic Mcl-1 is found in the cytoplasm or attached to the mitochondria and nuclear membrane in resting cells (Akgul *et al.*, 2000; Leuenroth *et al.*, 2000; Yang *et al.*, 1995). Unlike the other anti-apoptotic proteins, its localization is not solely dependent on its C-terminus TM domain (Akgul *et al.*, 2000; Chou *et al.*, 2006; Germain *et al.*, 2007). The C-terminus of Bcl-w is folded back and the protein can only loosely associate with the mitochondrial membrane; when apoptosis is induced, the C-terminal arm opens up and it is fully inserted into the membrane (Hinds *et al.*, 2003; Kaufmann *et al.*, 2004; Wilson-Annan *et al.*, 2003). Bcl-x_L is thought to act in a similar manner to Bcl-w; it is found in the cytoplasm or loosely attached to the mitochondrial membrane. In apoptotic cells, Bcl-x_L is inserted into the mitochondrial membrane. The subcellular location of Bcl-x_L is cell type specific.

Anti-apoptotic Bcl-2 is the first and best characterized Bcl-2 protein member, followed closely by Mcl-1. Both apoptotic inhibitors are discussed in greater detail in the following sections.

1.3.1.1.1.1 B-Cell Lymphoma (Bcl)-2

Yoshide Tsujimoto first identified the Bcl-2 gene in neoplastic cells in 1986 at the Croce laboratory (Tsujimoto *et al.*, 1984). Tsujimoto discovered that t(14:18) chromosomal translocation in follicular B-cell lymphomas augmented Bcl-2 levels. Bcl-2's apoptotic function, however, was only demonstrated a few years later by another laboratory that revealed a role for Bcl-2 in cell survival in leukemic cells (Reed *et al.*, 1990). Bcl-2 protein is essential for normal developmental in higher organisms. Bcl-2–deficient (Bcl-2 -/-) mice survive embryonic development, but display several detrimental features including growth retardation, early postnatal mortality, oxidative damage in kidneys, liver and brain, renal failure, and hypoplasia (Akao *et al.*, 1994; Fedorov *et al.*, 2006; Hochman *et al.*, 2000; Nagata *et al.*, 1996; Veis *et al.*, 1993). Overexpression of Bcl-2 has been extensively linked to many diseases, including autoimmune diseases such as Alzheimer's, arthritis, and nephritis, and to various cancers both solid and haematological malignancies including brain, breast, and lung cancer, CLL, and mantle cell lymphoma (Akao *et al.*, 1994; Kelly *et al.*, 2011; Masliah *et al.*, 1998; Tischner *et al.*, 2010). In contrast, reduced Bcl-2 levels have been observed in schizophrenic patients suggesting the induction of neuronal atrophy in the cerebral cortex of patients(Jarskog *et al.*, 2000).

1.3.1.1.1.2 Myeloid cell leukemia factor -1 (Mcl-1)

Mcl-1 was first identified in myeloblastic ML-1 cells, in which Mcl-1 levels were rapidly upregulated during differentiation (Kozopas *et al.*, 1993). Since its discovery, there has been growing interest in the role of Mcl-1. Mcl-1 is highly expressed in solid and haematological cancers and contributes resistance to standard chemotherapy (Derenne *et al.*, 2002; Hussain *et al.*, 2007; Paoluzzi *et al.*, 2008; Sieghart *et al.*, 2006).

Mcl-1 differs from the other anti-apoptotic proteins in its N-terminal region, and it has a relatively short half-life. The N-terminus mediates Mcl-1 ubiquitination, cleavage, and phosphorylation, which impacts the regulation and function of the protein. During apoptosis signaling, lysine residues located within the N-terminus of the Mcl-1 protein are ubiquinated and target Mcl-1 for ubiquitin–proteasomal degradation (Zhong *et al.*, 2005). Activated caspases can also cleave and degrade Mcl-1 in a ubiquitin-independent process (Clohessy *et al.*, 2004; Weng *et al.*, 2005). The first 79 amino acids (aa) of the N–terminal region localize Mcl-1 to the mitochondria; however, several regions situated throughout the protein structure also contribute to the subcellular localization of Mcl-1 (Akgul *et al.*, 2000; Chou *et al.*, 2006; Germain *et al.*, 2007). Additionally, Mcl-1 is phosphorylated on several serine and threonine phosphorylation sites within the N terminus that influence Mcl-1 stability, dimerization, and anti-apoptotic function (Ding *et al.*, 2007; Kobayashi *et al.*, 2007; Maurer *et al.*, 2006).

1.3.1.1.2 Pro-Death Subfamilies

Pro-death proteins of the Bcl-2 family can be categorized into two groups: pro-apoptotic (multi-domain) and BH3-only. The pro-apoptotic members are pore-forming proteins and modulate mitochondrial fragmentation. The BH3-only proteins regulate anti-apoptotic and/or pro-apoptotic proteins.

1.3.1.1.2.1 Pro-apoptotic Subfamily (Multi-domain)

Pro-apoptotic Bak, Bax and, Bok belong to the multi-domain subgroup (Youle *et al.*, 2008). They are regulated by anti-apoptotic proteins via inhibitory interactions that obstruct their oligomerization. Under normal conditions, Bak is localized to the OMM as an inactive monomer; it is stabilized in its inactive form by voltage-dependent anion channel (VDAC)-2. VDAC2 similarly binds inactive Bak

monomers and thus prevents both Bax- and Bak-dependent apoptosis (Cheng *et al.*, 2003). VDAC2 complex formation is required to keep Bax/Bak in inactive states. Inactive monomeric forms of Bax are also present in the cytosol where it associates with several proteins, including Ku-70 and 14-3-3. These interactions, however, are not necessary to keep Bax inactive. Bax and Bak remain dormant by tucking their C-terminal TM domains into their "BH grooves" (Yethon *et al.*, 2003). When apoptosis is activated, Bax and Bak change conformation and their N-terminus is exposed. These extensive conformational changes promote Bax/Bak homodimeric pore complex formation and trigger mitochondrial outer membrane permeabilization (MOMP); this irreversibly commits the cell to death (Wei *et al.*, 2001). Cytochrome c and other soluble proteins are rapidly released from the mitochondrial intermembrane space (IMS) following MOMP. Bax and Bak homo-oligomerization is crucial for permeabilization; mitochondrial dysfunction does not occur without it (Dewson *et al.*, 2008; George *et al.*, 2007). Recent studies have suggested that Bak is a critical mediator of mitochondrial fragmentation and Bax has a more prominent role in pore formation (Wei *et al.*, 2001).

Although Box shares extensive sequence homology to Bax and Bak, little is known about the pro-apoptotic protein (Inohara *et al.*, 1998). Box appears to have functional overlap with Bax and Bak, but it is more essential for the development of the female reproductive system (Ke *et al.*, 2012; Ray *et al.*, 2010).

Mitochondria are the powerhouse of the cell and provide all of the cell's energy (Frey *et al.*, 2000). They are also used as a storage site for free calcium, a key regulator of cellular homeostasis. Mitochondria are composed of an OMM, which is selectively permeable to proteins, and an inner mitochondrial membrane (IMM), which folds into invaginations called cristae. The mitochondria continuously undergo fission (division) and fusion (exchange of soluble proteins), which actively participate in apoptosis induction (Chan, 2007; Mitra *et al.*, 2009). Mitofusin (Mfn)1, Mfn2, and optic atrophy 1 (OPA1) are three large GTPases implicated in mitochondrial fusion (Meeusen *et al.*, 2006; Meeusen *et al.*, 2004; Santel *et al.*,

2001). The Mfn proteins mediate OMM fusion and the dynamin-related OPA1 mediates fusion of the IMM. Mitochondrial fusion proteins have recently been identified to actively induce apoptosis through interaction with key Bcl-2 family members. Co-immunoprecipitation studies reveal that both Mfn1 and Mfn2 form stable complexes with pro-apoptotic Bak (Brooks *et al.*, 2007; Suen *et al.*, 2008). In apoptotic cells, Bak dissociates from Mfn1 and the interaction between Bak and Mfn2 is greatly enhanced. These series of mitochondrial fusion events induce mitochondrial fragmentation within dying cells (Brooks *et al.*, 2007).

1.3.1.1.2.2 BH3-only Members

The BH3 domain, of the BH3–only members, is a nine-to 16-aa–long amphipathic α-helix that binds with high affinity to the hydrophobic groove on the surface of anti-apoptotic Bcl-2 proteins (Sattler *et al.*, 1997). BH3-only proteins have varying binding affinities for the anti-apoptotic proteins. Two distinct models of apoptosis induction have been proposed and they differ based on the role of the BH3-only proteins. In the first "direct" method, BH3-only members, namely Bim, Bid, and Puma, function as "activators," directly interacting with and activating Bax-like proteins at the mitochondria and trigger homo-oligomerization (Cartron *et al.*, 2004). The remaining BH3 members (i.e. Bad, Bik, and Hrk) reside in the cytoplasm and, upon apoptotic stimuli, act as "sensitizers." Sensitizers bind and neutralize anti-apoptotic Bcl-2 members, thus enhancing Bax/Bak activation and oligomerization, and mitochondrial membrane permeabilization. A clear distinction between sensitizers and activators is not always evident for each BH3-only protein, Noxa, for instance, has been shown to play a role in both subgroups (Dai *et al.*, 2011). In the second "indirect" mode of apoptosis, Bax and Bak remain inactive through inhibitory interactions with anti-apoptotic proteins (Willis *et al.*, 2007). When apoptosis is induced, BH3-only proteins bind anti-apoptotic members causing the release of both Bax and Bak. In this method, BH3-only proteins do not directly bind

Bax-like proteins. It has been suggested that both "direct" and "indirect" intrinsic apoptosis can occur within a cell and the mechanisms are not mutually exclusive (Merino *et al.*, 2009).

1.3.1.1.2.2.1 Bcl-2– Interacting Killer Protein (Bik)

Bik was the first BH3-only family member discovered. The human Bik gene is transcriptionally activated by transcription factor E2F and by p53. Once Bik is transcribed, coexpression of Noxa is required for it to mediate Bax activation and cytochrome c release (Willis *et al.*, 2005). Bik induces apoptosis via caspase-dependent and -independent mechanisms in many cancers (Naumann *et al.*, 2003; Oppermann *et al.*, 2005). Nonetheless, conflicting roles have been described for Bik during tumorigenesis. Bik is highly expressed in breast and non–small cell lung cancers; it is used as a prognosis marker and correlates with poor outcome (Lu *et al.*, 2006). On the other hand, Bik acts as a tissue specific tumor suppressor in severe combined immunodeficient mice (Daniel *et al.*, 1999).

1.3.1.1.2.2.2 Bcl-2–Associated Death Promoter (Bad)

Bad activity is negatively regulated by phosphorylation (Zha *et al.*, 1996). Dephosphorylation of Bad, at the serine-112 site, enhances its association with Bcl-2 and Bcl-$x_{L,}$ thus displacing Bax/Bak and promoting apoptosis (Scheid *et al.*, 1999; Zha *et al.*, 1996). Bad is negatively regulated by growth factors and cytokines that support cell survival (Kulik *et al.*, 1998). Bad is important for B-lymphocyte proliferation and IgG production; it also suppresses B-lymphoma growth (Ranger *et al.*, 2003).

1.3.1.1.2.2.3 Bcl-2 Interacting Mediator of Cell Death (Bim) and Bcl-2–Modifying Factor 1 (Bmf1)

Bim is able to bind to all the anti-apoptotic members. Alternate splicing of Bim generates three major Bim isoforms: (Bim(EL), Bim(L) and Bim(S); the latter is the most cytotoxic (O'Connor *et al.*, 1998). Bim expression is regulated at the transcriptional level by proteins such as forkhead transcription factor FoxO3A (Yang *et al.*, 2010). Bim is an important tumor suppressor and its expression is downregulated in B-cell non-Hodgkin lymphoma cancers (Mestre-Escorihuela *et al.*, 2007).

Humans have three splice variants of Bmf: BmfI (Bmf1), BmfII, and BmfIII (Morales *et al.*, 2004). The BmfII and BmfIII isoforms lack BH3 domains and do not contribute to apoptotic cell death like Bmf1. Bmf binds pro-survival proteins of the apoptotic family, permitting Bax/Bak oligomerization. Bmf1 is also induced under specific damage signals (eg. anoikis), in which cells detach from the extracellular matrix (Puthalakath *et al.*, 2001).

1.3.1.1.2.2.4 Phorbol-12-myristate-13-acetate-induced Protein 1 (PMAIP or Noxa) and p53-Upregulated Modulator of Apoptosis (Puma)

Pro-apoptotic PMAIP/ Noxa (meaning damage in Latin) is critical mediator of apoptosis (Oda *et al.*, 2000). In response to DNA damage, Noxa is transcriptionally regulated by p53 and IFN-regulatory factor (IRF)-3, although induction can occur in the absence of p53 (Goubau *et al.*, 2009; Oda *et al.*, 2000; Villunger *et al.*, 2003). Noxa contains a mitochondrial targeting domain within its C-terminus that targets the protein to the organelle (Seo *et al.*, 2003). In the mitochondria, Noxa binds with high specificity to Mcl-1 and Bfl-1/A1 (Chen *et al.*, 2005; Kuwana *et al.*, 2005). In certain cell types, Noxa targets Mcl-1 for proteasomal degradation in an attempt to positively regulate apoptosis (Nijhawan *et al.*, 2003).

Similar to Noxa, Puma is also dependently and independently regulated by p53 (Ming *et al.*, 2008; Villunger *et al.*, 2003; You *et al.*, 2006). Transcription factors FoxO3A, C/EBP homologous protein, and E2F1 upregulate Puma expression (Li *et*

al., 2006; You *et al.*, 2006). Like Bim, Puma interacts with all of the anti-apoptotic members and is thus localized to the mitochondria (Chipuk *et al.*, 2009). Puma's tumor suppressor activity is debatable, but it appears to be context-specific (Hemann *et al.*, 2004; Michalak *et al.*, 2008).

1.3.2 Apoptogenic Proteins

Intrinsic apoptosis perturbs the OMM and apoptogenic proteins such as cytochrome c, second mitochondrial activator of caspases/Direct inhibitors of apoptosis protein with low pI (Smac/DIABLO), HtrA2/Omi, apoptosis-inducing factors (AIF), and endonuclease G are released from the mitochondrial pore. These apoptogenic factors modulate distinct cellular activities. Cytochrome c is a small heme protein that associates with the OMM (Germain *et al.*, 2003). Cytochrome c is released from the within the mitochondria by the mitochondrial apoptosis-induced channel (MAC) (Dejean *et al.*, 2006). Upon its release, cytochrome c forms the apoptosome complex with apoptotic protease activating factor-1 (Apaf-1), adenosine-5'-triphosphate (ATP), and pro-caspase-9. Cytochrome c activation is a crucial step for caspase-3 cleavage (Liu *et al.*, 1996). EndoG is an endonuclease protein released from the IMS to the nucleus when it is primed for apoptosis. EndoG fragments DNA, but does so without the contribution of caspases (Li *et al.*, 2001; van Loo *et al.*, 2001). AIF, a mitochondrial intermembrane flavoprotein, which is also redirected to the nucleus upon activation, similarly induces DNA fragmentation and chromosome condensation in a caspase-independent manner (Kondo *et al.*, 2010; Susin *et al.*, 1999). Smac/DIABLO induces a caspase-dependent mechanism of cell death by suppressing inhibitors of apoptosis (IAPs) (Wilkinson *et al.*, 2004). HtrA2/Omi, a mitochondrial serine protease, also antagonizes IAPs (Gray *et al.*, 2000). HtrA2/Omi has spatial-specific functions; mitochondrial HtrA2 promotes cell survival, whereas cytosolic HtrA2/Omi stimulates both caspase-mediated and caspase-independent cell death mechanisms (Verhagen *et al.*, 2002).

1.3.3 Extrinsic Apoptosis

The mammalian death receptor pathway plays a pivotal role in the regulation and function of the immune system (Opferman *et al.*, 2003; Wilson *et al.*, 2009). Induction of the extrinsic pathway occurs at the cell surface where extracellular death ligands bind to pro-apoptotic death receptors (DR) (Figure 5). Death ligands (i.e. tumor necrosis factor (TNF), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL)) are produced by host immune cells (cytotoxic T lymphocytes, DC, macrophages, and NK cells) (Sayers, 2011). DRs oligomerize upon extracellular ligand binding transducing signals downstream (Boldin *et al.*, 1995). DRs are a subfamily of proteins belonging to the TNF receptor (TNFR) superfamily that contains a large number of proteins involved in developmental and differentiating processes (Ashkenazi *et al.*, 1998; Krammer, 2000; Nagata, 1997). There are currently six DRs characterized: TNFR1 (TNFRSF1A), Fas (also known as CD95, APO-1 or TNFRSF6), DR3 (TNFRSF12), DR4 (also known as TRAILR1 or TNFRSF10A), DR5 (also known as TRAILR2 or TNFRSF10B) and DR6 (TNFRSF21) (Gonzalvez *et al.*, 2010). The members of this family have type-I transmembrane proteins containing cysteine-rich extracellular domains (CRDs) in their N-terminal region. Apoptotic DRs exclusively contain homologous cytoplasmic death domains (DDs) that are necessary for caspase- and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-activating signal transmission. Upon receptor binding, the intracellular DDs associate with adaptor proteins — for instance TNF receptor–associated death domain (TRADD) and Fas-associated death domain (FADD), which bind TNFR1 and Fas, respectively. Inactive caspases are subsequently assembled and activated by the death-inducing signaling complex (DISC). DISC formation activates initiator caspase-8 and/or caspase-10, which is facilitated by death effector domains (DED) located in both the adaptor protein and the initiator caspases (Salvesen, 2002). Subsequently, effector caspase-3 and caspase-7, and Bid are cleaved and activated.

1.3.3.1 BH3 Interacting Domain Death Agonist (Bid)

Bid is a cytoplasmic BH3-only protein (Li *et al.*, 1998; Luo *et al.*, 1998). Unlike the other BH3 members, Bid facilitates extrinsic apoptotic signaling. Bid is cleaved into a truncated form by initiator caspase-8. Caspase-8 processing of Bid promotes crosstalk between the extrinsic death receptor and intrinsic mitochondrial apoptotic signaling pathways. This crosstalk serves to amplify the apoptotic signal. Truncated BID (tBid) binds to the OMM, promoting permeabilization and cytochrome c release from the mitochondria. At the OMM, tBID activates Bax and Bax; the presence of both proteins are vital for tBID-mediated apoptosis (Wei *et al.*, 2001). Recent work by Kaufmann et al. using Bid deficient mice revealed that Bid does not have a critical role in DNA damage response as cells undergo apoptosis without the protein (Kaufmann *et al.*, 2007).

1.3.4 Caspases

Caspases are a family of cysteine proteases that have been identified in lower organisms i.e. *Caenorhabditis elegans* to humans (Shi, 2002; Xue *et al.*, 1996). They regulate apoptosis-mediated cell death by cleaving their substrates after aspartate residues (Salvesen *et al.*, 1999; Shi, 2002; Xue *et al.*, 1996). Caspases exist in cells as an inactive form termed zymogens. There are two major types of caspases in humans: initiator caspases including caspase-2, -8, -9, and -10 and effector caspases inculding caspase-3, -6, and -7. Initiator caspases contain long N-terminal pro-domain regions that facilitate their interactions with other proteins. Structurally, initiator caspases have a caspase recruitment domain (CARD) (caspase-2 and -10) and/or DED (caspase-8 and -10) domains. Initiator caspases are activated by specialized caspase-activating complexes (Bao *et al.*, 2007). Effector caspases lack the CARD and DED domains and are activated by initiator caspases through proteolytic cleavage.

Caspase-independent forms of cell death have also been proposed; aided by apoptogenic proteins, AIF, endoG, and Omi/HtrA2 (Cande *et al.*, 2002; Comelli *et al.*, 2009; Pruefer *et al.*, 2008). Other proteases can also contribute to caspase-independent death, including granzymes, lysosomal cathepsins, matrix metalloproteinases, and proteasomal proteases (Lockshin *et al.*, 2004; Oppenheim *et al.*, 2001).

1.3.5 Caspase-Activating Complexes

Caspases are assembled and processed through the formation of various complexes, including the apoptosome, DISC, and the PIDDosome. Formation of the apoptosome, a 1.4 MDa complex, occurs during intrinsic signaling; the complex is assembled of seven Apaf-1 molecules, exogenously added dATP, and bound cytochrome c (Zou *et al.*, 1999). The Apaf-1 monomers contain three domains, an N-terminal CARD, a nucleotide-binding and oligormerization domain (NOD), and several WD40 repeats located at the C-terminus (Li *et al.*, 1997). The Apaf-1 CARD domain binds and activates procaspase-9. The WD40 repeats of Apaf-1 are required for cytochrome c binding. Upon cytochrome c binding and dATP addition, the Apaf-1 molecules oligomerize into a heptamer structure (Qin *et al.*, 1999). Apaf-1 has a prominent role in intrinsic apoptosis signaling; defects in the protein contribute to cancer progression (Umetani *et al.*, 2004).

Caspase activation through extrinsic signaling requires DISC assembly. DISC assembly is characterized by Fas, FADD, and pro–caspase-8 or -10 association, although other cofactors and regulatory proteins have also been implicated (Peter *et al.*, 2003). Fas ligand binding prompts the recruitment of FADD to the Fas receptor; FADD and caspase-8 interact through DED domains. The trimeric FasL and receptor can autoactivate up to three procaspase-8 molecules.

Stress-induced apoptosis stimulated by DNA damage occurs via the PIDDosome. The PIDDosome is a large, 670 kDa complex containing adaptor proteins RIP-associated Ich-1/Ced-3 homologous protein with a death domain (RAIDD) and PIDD (Tinel *et al.*, 2004). RAIDD contains both CARD and DD domains, which facilitate binding to initiator caspase-2 and PIDD, respectively (Park *et al.*, 2006). The exact mechanism of caspase-2 activation by the PIDDosome remains unknown.

1.3.6 Inhibitors of Apoptosis (IAPs)

IAPs are a conserved family of proteins that prevent cell death by physically binding caspases (Salvesen, 2002; Salvesen *et al.*, 2002; Vucic *et al.*, 2007). Eight subtypes of human IAPs have been described:

1) neuronal apoptosis inhibitory protein (also known as BIRC1),
2) cellular IAP1 (c-IAP1, also known as HIAP2, MIHB or BIRC2),
3) c-IAP2 (also known as HIAP1, MIHC, or BIRC3)
4) X-chromosome–linked IAP (XIAP, also known as hILP, MIHA, or BIRC4)
5) survivin (also known as TIAP or BIRC5)
6) apollon (also known as Bruce or BIRC6)
7) melanoma IAP (ML-IAP, also known as KIAP, livin, or BIRC7)
8) IAP-like protein 2 (also known as BIRC8, or Ts-IAP).

Caspase-3, -7, and -9 are negatively regulated by XIAP, c-IAP1 and c-IAP2 (Salvesen *et al.*, 2002). IAP function is blocked by apoptogenic factors (i.e. Smac/DIABLO and HtrA2/Omi) which shifts the cellular balance towards death (Wilkinson *et al.*, 2004). Aberrant expression and dysregulated IAP function is pronounced in many types of cancers (i.e. acute myeloid leukemia, acute lymphoblastic leukemia, and diffuse large B-cell lymphoma). Targeting of IAPs has

been suggested to improve the efficiency of several cancer treatments (Fulda, 2008; Smolewski *et al.*, 2011).

1.3.7 Apoptosis and CLL

Cellular homeostasis is the regulated balance of pro-death and pro-survival signals. Dysregulation of the apoptotic signaling pathway occurs in many types of diseases. Autoimmunity, cancer, Huntington's disease, and Alzheimer's disease have all been linked to defects in apoptosis (Kelly *et al.*, 2011; Masliah *et al.*, 1998; Tischner *et al.*, 2010). Characteristically, cancer cells are unable to undergo cell death. Pro-survival, Bcl-2, Mcl-1, and Bcl-X_L, are largely overexpressed in CLL patients and have prognostic importance (Buggins *et al.*, 2010). The accepted paradigm for CLL development is the accumulation of B cells defective in apoptosis as a direct result of the overexpression of anti-apoptotic proteins (Danilov *et al.*, 2006). The Bcl-2 promoter region is hypomethylated in CLL and is believed to correlate with high protein levels of Bcl-2 (Hanada *et al.*, 1993). micro-RNA (miR) species, miR-15, and miR-16, which have gene silencing activity, play a vital role in CLL development (Cimmino *et al.*, 2005). Both mIR species post-transcriptionally target Bcl-2 and enhance apoptotic signaling in transfected human megakaryocytic cells. miR-15 and miR-16 expression levels are substantially downregulated or completely deleted in many CLL patients, although an inverse correlation between miR-15, miR-16, and Bcl-2 protein expression has also observed in CLL cells. p53 mutations and loss of p53 function is an important factor in apoptosis dysregulation. p53 induces the expression of several pro-apoptotic proteins (i.e. Puma, Noxa, Bax) in addition to inhibiting the transcription of pro-survival proteins such as Bcl-2 (Schuler *et al.*, 2001). Mutations in p53 promote defective apoptosis signaling in CLL. Collectively, these findings highlight the need for therapeutics that target the apoptotic pathway in CLL (Miyashita *et al.*, 1995; Oda *et al.*, 2000; Yu *et al.*, 2003).

1.4 Macroautophagy (Autophagy)

Macroautophagy (referred to herein as autophagy) is a ubiquitous mechanism that non-selectively sequesters and degrades organelles for bulk degradation using the cell's own lysosomal compartment (Levine *et al.*, 2004). Cytosolic material is engulfed into large double-membrane vesicles termed autophagosomes. Autophagy induction is elicited by a number of varying stimuli, including starvation, hypoxia, extreme temperatures, overpopulation, damaged organelles and unnecessary cytoplasmic components.

The earliest record of autophagy was in 1957 when Sam Clark identified "irregular cytoplasmic inclusions" that contained various cellular structures (Clark, 1957). The name was coined in the early 60s by Christian de Duve, a founding father of autophagy, who discovered that glucagon, a hormone secreted from pancreatic cells, induced the formation of lysosomal vesicles (Deter *et al.*, 1967). Autophagy is a Greek term meaning eat ("phagy") oneself ("auto"). As the Greek name implies, autophagy is a self-digesting process, but it is also important in survival, cellular remodeling, and recycling (Levine *et al.*, 2004; Mizushima, 2007). Within recent years, the field of autophagy has increased exponentially with growing interest into its role in immunity, viral pathogenesis, and disease development. Many forms of autophagy have been identified, including macroautophagy, chaperone-mediated autophagy and microautophagy (Levine *et al.*, 2004).

1.4.1 Autophagy Process and Function

The multistep process of autophagy is conserved from *Saccharomyces cerevisiae* (yeast) to humans (Mizushima, 2007; Mizushima *et al.*, 2010). Autophagy induction occurs in response to various triggers. Upon induction, organelle-

containing cytoplasm is sequestered into a small vesicular sac known as the phagophore (a.k.a. isolation membrane). The phagophore elongates into an autophagosome (Figure 7). The outer membrane of the autophagosome fuses with the endosome and maturates into amphisomes. Amphisomes subsequently fuse with the lysosome generating autophagolysosome (a.k.a autolysosome), where the intravacuolar components are degraded.

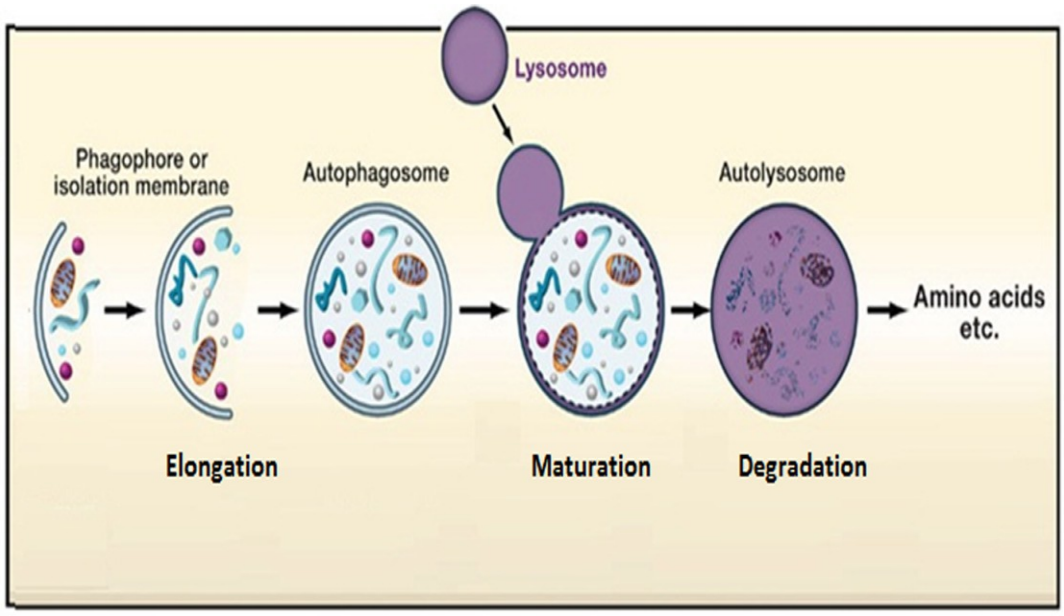

Figure 7: Autophagosome Formation.

The autophagy process involves the engulfment of cytoplasmic material into an isolation membrane/phagophore which elongates, forming a double membrane autophagosome. During maturation, the autophagosome fuses with endosomes to form amphisomes which subsequently fuse with lysosomes to form autolysosomes/autophagolysosome. The contents of the autolysosome are degraded by hydrolytic enzymes.

(Adapted with permission from (Mizushima *et al.*, 2010)).

1.4.1.1 Induction of Autophagy

Autophagy is induced in response to multiple distinct stimuli. Nutrient starvation is one of the major triggers of the autophagic process. The pathway is also stimulated by various cellular factors, including aa depletion; general control nonrepressor 2 (GCN2), a eukaryotic initiation factor-2-α; reactive oxygen species, TRAIL; and glucagon; it is negatively regulated by Bcl-2, insulin, hematopetic growth factor, and IL-3 (Mizushima, 2007; Mortimore *et al.*, 1983; Talloczy *et al.*, 2002). Most of the cellular factors that influence autophagy regulation do so through the mammalian target of rapamycin (mTOR) signaling pathway, a prominent regulator of nutrient-stimulated signaling. Inhibition of the mTOR pathway activates autophagy in both yeast and mammals; however mTOR-independent mechanisms of autophagy have been described (Mizushima, 2007). For instance, muscle cells depleted of leucine activated autophagy even when mTOR was pharmacologically inhibited (Mordier *et al.*, 2000).

1.4.1.2 Elongation

During elongation of the phagophore, cytoplasmic material taken up for degradation is sequestered into a phagophore vacuole (Mizushima *et al.*, 2002). The phagophore associates with a number of proteins or protein complexes, elongates, and produces a double-membrane autophagosome vesicle. The preautophagosomal structure (PAS) is the earliest precursor complex identified for the autophagosome (Kim *et al.*, 2002; Suzuki *et al.*, 2001). It was discovered in yeast and, to date, an equivalent structure has not been identified in humans. A group of autophagy-related genes (Atgs) were also discovered in yeast (Levine *et al.*, 2004). Atgs facilitate autophagy-specific cargo selection, vesicle expansion, membrane fusion, etc. Several

of the yeast Atgs have also been identified in higher eukaryotes and have similar functions.

In mammalian cells, the Atg proteins coordinate several processes during elongation. In the early stages of elongation, the phagophore associates with the uncoordinated (Unc)-51-like kinase 1 (ULK1):Atg13:focal adhesion kinase family interacting protein of 200 (FIP200) complex (ULK-1:Atg13:FIP200) (Mehrpour *et al.*, 2010). This association is prevented when the mTOR kinase pathway phosphorylates ULK1 and/or Atg13 (Ganley *et al.*, 2009). Another Atg protein, Atg101, is a critical regulator of autophagy (Hosokawa *et al.*, 2009; Mercer *et al.*, 2009). Atg101 interacts with Atg13 and ULK1 and is responsible for the basal phosphorylation and stability of Atg13 and ULK1, which prevents proteasomal degradation of the proteins. Basal phosphorylation of Atg13 and ULK1 by Atg101 positively regulates autophagy. When Atg101 protein levels are knocked downed in cells autophagosome formation is inhibited (Hosokawa *et al.*, 2009). Another complex containing Beclin 1, vacuolar protein sorting (Vps) 34 and Atg14L (Beclin-1:Vps34:Atg14L), is involved in nucleation and assembly of the phagophore membrane (Mehrpour *et al.*, 2010). Vps34 is a Class III phosphoinositide 3-kinase (PI3K) that produces phosphatidylinositol 3-phosphate (PtdIns(3)P) (Axe *et al.*, 2008; Backer, 2008). PtdIns(3)P is incorporated into the phagophore membrane and is important for membrane trafficking and phagosome maturation. Beclin-1 a.k.a Atg6 regulates PtdIns(3)P production by Vsp34 (Wirawan *et al.*, 2012). It also recruits other Atg proteins to the autophagosome to aid in elongation. Atg9L1 is a transmembrane protein that cycles between the trans-Golgi network and the endosome (Takahashi *et al.*, 2011). The only suggested role for Atg9L1 is to transport lipids and proteins necessary for autophagosome formation (Yoshimori *et al.*, 2008).

Seven mammalian proteins participate in the final stages of elongation. Atg7 activates Atg12 and forms an intermediate complex with the protein (Mizushima *et al.*, 2002; Tanida *et al.*, 2002). Atg12 is then transferred to the Atg10 and in a final

transfer step, Atg12 is conjugated to Atg5 and the two proteins are constitutively linked. The Atg12-Atg5 complex associates with Atg16L which can interact with additional proteins through its WD40 repeat domain (Mizushima *et al.*, 2003). Although most of the Atg12-Atg5-Atg16L complex is present in the cytosol, a small fraction of this complex binds the phagophore and remains with the vacuole until the autophagosome is completely formed. Microtubule-associated protein light chain 3 (MAP-LC3 or LC3) protein associates with the autophagosome membrane after a series of post-translational modifications carried out by Atg4 and Atg3 (He *et al.*, 2003; Tanida *et al.*, 2002). In the cytosol, Atg4 proteolytically cleaves LC3, leaving a C-terminal glycine residue forming what is known as LC3-I. An Atg3-dependent ubiquitination-like enzymatic reaction lipidates LC3-I with phosphatidylethanolamine (PE), converting it into LC3-II (Kabeya *et al.*, 2000). LC3-II localizes with the outer and inner autophagosome membrane and remains there after its formation (Yamaguchi *et al.*, 2010). LC3-II is the only known structural protein to permanently remain with the autophagosome membrane, thus it is a key protein to identify experimentally to measure autophagosome formation.

1.4.1.3 Maturation

During maturation, amphisome vacuoles are generated from autophagosomes that are fused to early and late endosomes or to multivesicular bodies (Mizushima, 2007; Mizushima *et al.*, 2010). The maturation process is regulated by several key proteins, including Run domain protein as Beclin 1 interacting and cysteine-rich containing (Rubicon), UV irradiation resistance-associated gene (UVRAG), Ras-related GTP-binding protein (Rab)7, Endosomal sorting complex required for transport (ESCRT)–III, Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), Vesicle-associated membrane protein (VAMP)3, VAMP7, Lysosomal-associated membrane proteins (LAMPs), and Damage-regulated autophagy modulator (DRAM)-2.

Both Rubicon and UVRAG can bind Beclin-1 when it is complexed with Vps34 and Atg14L, but the proteins have opposing functions (Liang *et al.*, 2006; Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). UVRAG is a positive regulator of the Beclin 1 complex, and it has been identified as a possible tumor suppressor in human colon cancer cell lines. Rubicon, on the other hand, negatively regulates autophagy through Beclin-1 binding. Rab7 is a small GTPase belonging to the Rab family that regulates endosomes trafficking and lysosome biogenesis; it is activated by UVRAG (Jager *et al.*, 2004). When Rab 7 is knocked down in HeLa cells, LC3-II is no longer formed and autophagosome maturation is delayed. ESCRT–III and Hrs mediate protein sorting in multivesicular bodies, endosomes, and lysosomes (Raiborg *et al.*, 2009). Mutations in ESCRT-III have been linked to susceptibility to amyotrophic lateral sclerosis and frontotemporal dementia (Parkinson *et al.*, 2006; Skibinski *et al.*, 2005). Two members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family of fusion proteins are involved in maturation, VAMP3, and VAMP7. VAMP3 is required for amphisome formation and VAMP7 for lysosomal fusion (Fader *et al.*, 2009). Two structurally related LAMPs (transmembrane glycoproteins), LAMP-1 and -2, have overlapping roles in autophagosome maturation (Eskelinen *et al.*, 2003). The LAMP proteins participate in enzyme targeting and lysosomal biogenesis. Lastly, DRAM-2 is a p53-regulated protein located within the lysosome (Yoon *et al.*, 2012). The exact function of DRAM2 is not well known, but silencing of DRAM2 expression reduces autophagosome formation.

1.4.2 Degradation

During the final step of autophagy, the autophagosome fuses with lysosome-forming autophagolysosomal complexes (Mehrpour *et al.*, 2010). Formation of the autophagolysosome results in the acidic breakdown and recycling of engulfed components. Vacoular ATPases (v-ATPases) break down ATP to adenosine diphosphate (ADP), which drives proton translocation into the vacuole and increases

acidification of the lysosome compartment (Mijaljica *et al.*, 2011). Other luminal hydrolyases are activated as a result of the drop in pH and break down internalized material. Finally, there is an efflux of lysosomal contents into the cytosol where the material can be recycled for further use (Lloyd, 1996).

1.4.3 Autophagic Cell Death

Autophagic cell death is a second form of programmed cell death (the first being apoptosis), although its existence and prevalence is highly debated (Shen *et al.*, 2011; Shen *et al.*, 2012). Autophagosomes and other autophagic structures are present in dying cells and this suggests that an alternate form of programmed cell death is induced by autophagy (Tsujimoto *et al.*, 2005). It has been argued that autophagy accompanies other forms of death rather than directly causing cell death (Shen *et al.*, 2011). Because autophagy promotes survival, it can be activated in an attempt to save dying cells. Nevertheless, autophagy is induced in caspase- and necrosis-independent cell deaths, and under these circumstances, it is directly responsible for cell destruction. Moreover, it has been suggested that the Bcl-2 apoptotic family mediates autophagic cell death through Beclin-1 regulation (Maiuri *et al.*, 2007; Pattingre *et al.*, 2005; Shimizu *et al.*, 2004; Yu *et al.*, 2004).

1.4.4 Bcl-2 Regulation of Autophagy

Beclin-1 was the first identified mammalian autophagy gene product and is a haplo-insufficient tumor suppressor (Sinha *et al.*, 2008). Beclin-1 is a novel BH3 domain containing protein, but unlike the other BH3-only members, Beclin-1 functions in autophagy rather than apoptosis. Anti-apoptotic proteins Bcl-2, Bcl-$x_{L,}$ and Mcl-1 disrupt autophagy induction through direct inhibition of Beclin-1 (Elgendy *et al.*, 2011; Maiuri *et al.*, 2007; Pattingre *et al.*, 2005). Bcl-2, Bcl-x_L, and Mcl-1 are commonly overexpressed in many cancers and function to dysregulate autophagy and apoptotic signaling (Akgul, 2009; Gordy *et al.*, 2012; Hockenbery,

2010). Bcl-2 regulation of autophagy creates a crosstalk with the apoptotic signaling pathway (Kang *et al.*, 2011). This crosstalk is facilitated by apoptotic BH3-only proteins, Bad and Noxa, which abrogate inhibitory Bcl-2/Bcl-x_L/Mcl-1:Beclin-1, interactions and by phosphorylated Bcl-2, which releases Beclin-1 (Elgendy *et al.*, 2011; Maiuri *et al.*, 2007; Wei *et al.*, 2008). Other methods of crosstalk have also been identified (Gordy *et al.*, 2012; Rambold *et al.*, 2011).

1.4.5 Autophagy, Viral Infection and Immunity

Autophagy is largely thought of as a non-selective mechanism of bulk degradation that is induced by starvation, however it also selectively targets intracellular pathogens and helps mount an immune response to infection (Levine *et al.*, 2011). Selective targeting of organelles and pathogens results in many forms of autophagic degradation such as xenophagy (intracellular virus bacteria, fungi and/or parasite degradation), mitophagy (mitochondria degradation), pexophagy (peroxisomes degradation), ER-phagy (ER degradation), ribophagy (ribosome degradation) to name a few (Klionsky *et al.*, 2007). The exact mechanism of virus xenophagy is not fully understood, and autophagy can either target a virus for death or promote its replication and survival (Orvedahl *et al.*, 2009). Viruses have evolved several evasion mechanisms to counteract autophagy.

1.4.5.1 Autophagy in Antiviral Defense

Xenophagy plays a protective role in antiviral immune defense. Viral replication is diminished in the presence of autophagy protein Beclin-1. Sindbis virus-mutants expressing a full length form of autophagy protein Beclin-1 replicate less efficiently in the brains of infected mice in comparison to mice infected with a truncated form of Beclin-1 (Liang *et al.*, 1998). Studies by Orvedahl *et al.*

demonstrated that HSV 1 neurovirulence protein, ICP34.5, confers resistance to autophagy through inhibitory binding of Beclin-1 and mice die by lethal encephalitis (Orvedahl *et al.*, 2007). Mutant HSV 1 that is unable to bind Beclin-1 are neuroattenuated *in vivo*. Taken together, these results support a protective role for autophagy in viral infection. It is clear however that many viruses have devised mechanisms to inhibit autophagy induction.

Autophagy also mediates innate and adaptive immune responses to viral infection (Levine *et al.*, 2007). Xenophagic degradation produces viral nucleic acid and protein products that are used to activate the innate and adaptive immunity. Viral components are sensed by plasmacytoid DC through toll-like receptor-7, and IFN-α-mediated innate immune responses are thus activated (Lee *et al.*, 2007). Viral products are also presented on major histocompatibility class II molecules to T cells which specifically activate adaptive immunity (Gannage *et al.*, 2009).

1.4.5.2 Autophagy-Enhanced Viral Replication

Although autophagy is known for its antiviral defense mechanism, in a contrasting approach, autophagy can also enhance replication of certain viruses, and in a few cases it can inhibit innate immune signaling thus promoting pathogenesis. Some viruses utilize the autophagic machinery to enhance replication, for instance VSV replication is dependent upon the autophagy machinery (Jounai *et al.*, 2007). During VSV infection, the Atg5-Atg12 complexes inhibit the innate immune response by direct blocking antiviral response proteins, retinoic acid-inducible gene I (RIG-I) and IFN-β promoter stimulator 1 (IPS-1). The autophagosome complex (Atg5-Atg12) is able to bind and inhibit these innate antiviral response proteins through equivalent CARD domains. Additionally, Atg5 and Atg7 encourage viral replication by repressing type I IFN production. Autophagy-dependent replication has also been reported for poliovirus and coxsackievirus where autophagy is used as a scaffold for viral replication (Jackson *et al.*, 2005; Wong *et al.*, 2008). Poliovirus

and coxsackievirus infection induce autophagy and viral reproduction complexes associate with the autophagosome facilitating viral replication and protein expression. It is hypothesized that this association also facilitates the nonlytic release of virions along with cytoplasmic components from the autophagolysosome upon autophagic degradation.

1.4.6 Autophagy and Cancer

The contrasting roles autophagy plays in viral pathogenesis carries over to its role in cancer development; it is often referred to as having a "Dr. Jekyll/Mr. Hyde-like" personality (Shen *et al.*, 2011). Autophagy enhances or inhibits tumorigenesis in a context-specific manner (Shen *et al.*, 2011; White, 2012).

When autophagy is induced to inhibit tumor formation, the pathway promotes oncogene-induced cell death and aids in the removal of harmful proteins and damaged organelles (White, 2012). Beclin-1, Atg5, and Atg7 expression is critical for tumor suppression in certain cancer subtypes. For example, double knockout of Beclin-1 in mice cause early embryonic lethality (Qu *et al.*, 2003; Yue *et al.*, 2003). Beclin-1+/- mice survive the embryonic stage, but develop spontaneous tumors and hepatitis B virus–induced premalignant lesions and neoplasms. Deficiencies in Atg5 and Atg7 also induce cancer formation (Takamura *et al.*, 2011).

In an opposite manner, autophagy is activated by cancer cells in an attempt to prolong cell survival. Autophagy regulates the maintenance of mitochondrial metabolism and this can lead to autophagy-dependent tumor development (Guo *et al.*, 2011). Autophagy is also activated by cancer cells in response to the harsh microenvironmental conditions that arise during tumor development as a result of starvation, growth factor depletion, and hypoxia (Degenhardt *et al.*, 2006). Nutrient levels diminish in cancer cells with overactivated Ras signaling, and autophagy is induced to be able to survive nutrient depletion (Guo *et al.*, 2011). In Ras-activated

cancers, knockdown of Atg5 and Atg7 actually suppresses cancer growth. Primary pancreatic cancer tumors have high basal autophagy levels (Yang *et al.*, 2011). Tumor growth can be inhibited in pancreatic cancers by pharmacological inhibition of autophagy or Atg5 gene silencing. Together, these results highlight the context-dependent regulation of autophagy during tumorigenesis.

1.5 Bcl-2 Inhibitors

The balance between the pro- and anti-apoptotic Bcl-2 family members is a major determinant of cell survival or death. The recent implication of the pro-survival Bcl-2 proteins in autophagy regulation also emphasizes the importance of the apoptotic family in normal autophagy function. It is not surprising that disruption of Bcl-2 regulation of cellular homeostasis can result in the development of cancer. Pro-survival Bcl-2 proteins are not only overexpressed in many cancers, but they also contribute to chemoresistance (Chao *et al.*, 1998; Chao *et al.*, 1995; Minn *et al.*, 1995; Pepper *et al.*, 1998). Over the past decade, research has led to the development of small-molecule Bcl-2 inhibitors as a means to circumvent resistance to therapy (Bajwa *et al.*, 2012). These novel anti-cancer drugs are designed to mimic BH3-only protein; they bind to anti-apoptotic members and allow the release of pro-apoptotic proteins (i.e. Bax, Bak, Bim, and Bid) and pro-autophagic Beclin-1, thus restoring order to both the apoptotic and autophagic pathways (Figure 8). Mechanistically, Bcl-2 inhibitors are hypothesized to bind the hydrophobic BH3-binding groove of pro-survival proteins. Bcl-2 inhibitors represent a promising class of anti-cancer therapeutics, and many of the compounds are being tested clinically in phase I–III trials.

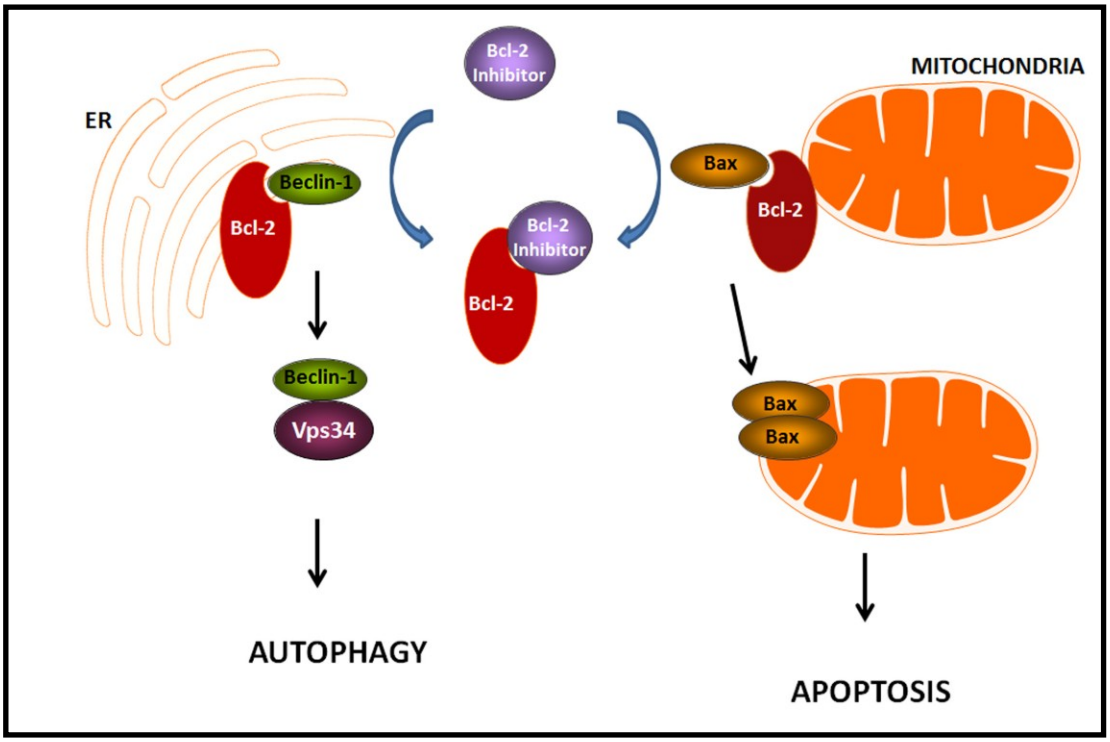

Figure 8: Bcl-2 inhibitors.

Bcl-2 inhibitors are small molecule BH3 mimetics that antagonize anti-apoptotic proteins at the mitochondria and ER allowing the release of Bax-like proteins and Beclin-1. Bcl-2 inhibitors activate both apoptotic and autophagic cell signaling pathways.

ER: Endoplasmic Reticulum, **Vps:** Vacuolar protein sorting.

Adapted with permission from (Notte *et al.*, 2011).

1.5.1 Obatoclax (GX15-070)

Obatoclax (GX15-070) is a Canadian-developed Bcl-2 inhibitor designed by Gemin X Biotechnologies (Montreal, QC). It is a derivative of synthetic prodiginine, a family of pigments with immunosuppressant and anti-cancer and antiviral properties (Gerber, 1975). Obatoclax is a pan–Bcl-2 inhibitor, targeting all members of the anti-apoptotic subfamily within a 1 to 7 micromolar (μM) range (Shore *et al.*, 2005; Zhai *et al.*, 2006). Obatoclax is especially important for its use in cancers with upregulated Mcl-1, as other inhibitors such as ABT-737 do not target this protein (Mazumder *et al.*, 2012; Nguyen *et al.*, 2007). Mechanistically, Obatoclax activates apoptosis in a Bax-mediated manner in cholangiocarcinoma cells. Small hairpin RNA knockdown of Bax significantly decreased cell susceptibility to Obatoclax-mediated death (Smoot *et al.*, 2010). Studies in acute myeloid leukemia primary cells revealed a Bax-independent pathway of apoptosis, Obatoclax-mediated Bak and Bim release from Mcl-1 and Bcl-2 without Bax activation (Konopleva *et al.*, 2008). Obatoclax can also disrupt Mcl-1:Beclin-1 complexes, inducing an autophagy-dependent cell death mechanism (Bonapace *et al.*, 2010).

Obatoclax is effective as a single-agent treatment in many cancer cell lines, including non–small cell lung cancer, mantle cell lymphoma, multiple myeloma, and *ex vivo* CLL primary cells (Li *et al.*, 2008; Perez-Galan *et al.*, 2007; Shore *et al.*, 2005; Trudel *et al.*, 2007). Obatoclax is currently being tested in phase I/II clinical trials as both a single agent or combination therapy in refractory leukemia, myelodysplasia, CLL, colon cancer, non-Hodgkin's lymphoma, and small-cell lung cancer patients as well as other solid and hematological malignancies (Hwang *et al.*, 2010; O'Brien *et al.*, 2009; Paik *et al.*, 2010; Schimmer *et al.*, 2008). The inhibitor is well tolerated in many patient groups; however, Obatoclax fails to reach maximum efficacy when delivered intravenously, and neurological dose-limiting toxicity was observed in some cancer patients. In aggressive CLL patients who previously

received chemotherapy and antibody-based therapies, single treatment with Obatoclax appears to be limited (O'Brien *et al.*, 2009).

1.5.2 ABT-737 and ABT-263 (Navitoclax)

Bcl-2 inhibitor ABT-737, and its orally active analog ABT-263 (Navitoclax), were developed by Abbott Laboratories. The ABT compounds have some of the highest affinities for pro-survival Bcl-2 proteins, achieving cytotoxicty at nanomolar concentrations (Tse *et al.*, 2008; Zhai *et al.*, 2006). ABT-737 and ABT-263 were discovered using structure-activity relationship (SAR) by nuclear magnetic resonance (NMR)-based screening methods (Oltersdorf *et al.*, 2005). ABT-737 was designed to mimic the binding capabilities of pro-apoptotic Bad; therefore, the agents bind with high affinity to Bcl-2, Bcl-x_L, Bcl-$_W$, and with very low affinity to Mcl-1 and Bfl-1. ABT-737 can only bind Mcl-1 and Bfl-1 in the μM range because the obstructed BH3 groove conformation within the proteins possibly deters binding (Day *et al.*, 2005; Oltersdorf *et al.*, 2005). Consequently, ABT-737 has displayed limited efficacy as a single-agent treatment for cancers with Mcl-1 overexpression (Mazumder *et al.*, 2012). ABT-737 enhances apoptosis in primary acute lymphoblastic leukemia, acute myeloid leukemia, and CLL cells (Del Gaizo Moore *et al.*, 2007; Del Gaizo Moore *et al.*, 2008; Konopleva *et al.*, 2006). ABT-737 induces apoptosis in a Bak/Bak-dependent mechanism (van Delft *et al.*, 2006). The inhibitor can also induce Beclin-1-dependent autophagy through Bcl-2 inhibition (Maiuri *et al.*, 2007).

The orally active ABT-263 has similar binding properties to ABT-737; small-cell lung carcinoma (SCLC), leukemic and lymphoma cells are sensitive to single-agent ABT-263 treatment (Tahir *et al.*, 2010). These studies also revealed Mcl-1–mediated resistance. ABT-263 is currently being investigated in phase I/II clinical trials in SCLC and CLL (Gandhi *et al.*, 2011; Roberts *et al.*, 2012). Thrombocytopenia is the major dose-limiting factor in patients. Abbott Laboratories

has recently developed another inhibitor, ABT-199, proposed to bind with higher affinity to Bcl-2 and offset the thrombocytopenia caused by their other compound (Bajwa *et al.*, 2012; Davids *et al.*, 2012).

1.5.3 Gossypol and Analogues

Gossypol (BL-193) was the first Bcl-2 inhibitor identified. It works by antagonizing Bcl-2, Bcl-$x_{L,}$ and Bcl-$_W$. Gossypol is a naturally occurring compound derived from the cotton plant. BL-193 was the first inhibitor tested clinically even though its mechanism of action was not known at the time (Bushunow *et al.*, 1999). Levo-gossypol ((−)-gossypol, AT-101), a natural enantiomer of gossypol, was developed by Ascenta Pharmaceuticals with submicromolar affinities for Bcl-2 and Mcl-1. The compound is used in phase I–III trials for the treatment of CLL, adrenocortical carcinoma, and prostate cancer, but causes dose-limiting gastro-related toxicity (Bajwa *et al.*, 2012; MacVicar GR, 2008). Gossypol and its enantiomers activate intrinsic apoptotic signaling through a variety of methods (Balakrishnan *et al.*, 2009; Macoska *et al.*, 2008; Niu *et al.*, 2012). An apoptosis-independent Beclin-1 and Atg-5–mediated cell death is described in prostate and glioma cancer cells treated with AT-101 (Lian *et al.*, 2010; Voss *et al.*, 2010). Apogossypol (ApoG2) is a third-generation derivative of gossypol developed by Ascenta Pharmaceuticals, and was designed to address the clinical toxicity issues with gossypol (Azmi *et al.*, 2011). The compound is being tested pre-clinically.

TW-37 is a benzenesulfonyl derivative of gossypol discovered using structure-based design at the University of Michigan (Wang *et al.*, 2006). TW-37 negatively regulates Bcl-2, Mcl-1, and Bcl-x_L within nanomolar concentrations. The compound stimulates apoptosis and inhibits tumor angiogenesis in prostate cancer, and head and neck squamous cell carcinoma (Ashimori *et al.*, 2009; Wang *et al.*, 2006). TW-37 is currently in pre-clinical trials only.

1.5.4 HA14-1

HA14-1 (ethyl-2-amino-6-cyclopentyl-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromone-3-carboxylate) was discovered using a computer screening strategy based on the BH3 binding groove of Bcl-2 (Wang *et al.*, 2006). HA14-1 specifically targets Bcl-2 in the μM range. This compound is able to inhibit structurally related Bcl-x_L protein in human glioblastoma cells (Manero *et al.*, 2006). HA14-1 synergistically activates Bax-mediated apoptosis in human glioblastoma cells when combined with radiotherapy and chemotherapy. The inhibitor also sensitizes oesophageal cancer cells to apoptosis-deficient autophagy-associated cell death (Nyhan *et al.*, 2012). HA14-1 is currently not in clinical trials.

1.5.5 Antimycin A

Antimycin A is an anti-fungal *Streptomyces*-derived compound. The compound, a known inhibitor of mitochondrial electron transfer, was also discovered to antagonize Bcl-x_L and Bcl-2, and displaces Bak (Bajwa *et al.*, 2012; Tzung *et al.*, 2001). Antimycin A inhibits growth of human cervical adenocarcinoma cells *in vitro*, but high concentrations are required for *in vivo* efficacy (Park *et al.*, 2007). In contrast to the other Bcl-2 inhibitors, Antimycin A blocks autophagy induction (Ma *et al.*, 2011). Antimycin A is being tested pre-clinically (Bajwa *et al.*, 2012).

1.5.6 Therapeutic Implications

In recent years, there has been increasing interest in identifying novel Bcl-2 inhibitors for the treatment of various malignancies, including solid and hematological cancers. These BH3 mimetics are identified by many high throughput screening processes designed to increase binding affinity and specificity. Obatoclax and gossypol are the only pan–Bcl-2 inhibitors in clinical trials. The effectiveness of

other inhibitors such as ABT-737 and ABT-263, are hampered by Mcl-1–conferred resistance. Although many inhibitors are effective as single agents in a variety of cancers, improved results are achieved in combination therapies. Dose-limiting toxicities occur in many patients and neuro- and gastro-toxicity has been reported in addition to low platelet counts (thrombocytopenia). Many companies are designing novel inhibitors with reduced side effects. However, low-dose combination therapy might be a better alternative.

References

Ahmed, M., & Lyles, D. S. (1998). Effect of vesicular stomatitis virus matrix protein on transcription directed by host RNA polymerases I, II, and III. *J Virol, 72*(10), 8413-8419.

Ahmed, M., McKenzie, M. O., Puckett, S., Hojnacki, M., Poliquin, L., & Lyles, D. S. (2003). Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. *J Virol, 77*(8), 4646-4657.

Akao, Y., Otsuki, Y., Kataoka, S., Ito, Y., & Tsujimoto, Y. (1994). Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. *Cancer Res, 54*(9), 2468-2471.

Akgul, C. (2009). Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cell Mol Life Sci, 66*(8), 1326-1336. doi: 10.1007/s00018-008-8637-6

Akgul, C., Moulding, D. A., White, M. R., & Edwards, S. W. (2000). In vivo localisation and stability of human Mcl-1 using green fluorescent protein (GFP) fusion proteins. *FEBS Lett, 478*(1-2), 72-76.

Anderson, B. D., Nakamura, T., Russell, S. J., & Peng, K. W. (2004). High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus. *Cancer Res, 64*(14), 4919-4926. doi: 10.1158/0008-5472.CAN-04-0884

Andritsos, L., & Khoury, H. (2002). Chronic lymphocytic leukemia. *Curr Treat Options Oncol, 3*(3), 225-231.

Ashimori, N., Zeitlin, B. D., Zhang, Z., Warner, K., Turkienicz, I. M., Spalding, A. C., Teknos, T. N., Wang, S., & Nor, J. E. (2009). TW-37, a small-molecule inhibitor of Bcl-2, mediates S-phase cell cycle arrest and suppresses head and neck tumor angiogenesis. *Mol Cancer Ther, 8*(4), 893-903. doi: 10.1158/1535-7163.MCT-08-1078

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science, 281*(5381), 1305-1308.

Axe, E. L., Walker, S. A., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., Griffiths, G., & Ktistakis, N. T. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol, 182*(4), 685-701. doi: 10.1083/jcb.200803137

Azmi, A. S., Wang, Z., Philip, P. A., Mohammad, R. M., & Sarkar, F. H. (2011). Emerging Bcl-2 inhibitors for the treatment of cancer. *Expert Opin Emerg Drugs, 16*(1), 59-70. doi: 10.1517/14728214.2010.515210

Backer, J. M. (2008). The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem J, 410*(1), 1-17. doi: 10.1042/BJ20071427

Bajwa, N., Liao, C., & Nikolovska-Coleska, Z. (2012). Inhibitors of the anti-apoptotic Bcl-2 proteins: a patent review. *Expert Opin Ther Pat, 22*(1), 37-55. doi: 10.1517/13543776.2012.644274

Balakrishnan, K., Burger, J. A., Wierda, W. G., & Gandhi, V. (2009). AT-101 induces apoptosis in CLL B cells and overcomes stromal cell-mediated Mcl-1 induction and drug resistance. *Blood, 113*(1), 149-153. doi: 10.1182/blood-2008-02-138560

Ball, L. A., Pringle, C. R., Flanagan, B., Perepelitsa, V. P., & Wertz, G. W. (1999). Phenotypic consequences of rearranging the P, M, and G genes of vesicular stomatitis virus. *J Virol, 73*(6), 4705-4712.

Bao, Q., & Shi, Y. (2007). Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ, 14*(1), 56-65. doi: 10.1038/sj.cdd.4402028

Barr, J. N., Whelan, S. P., & Wertz, G. W. (2002). Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Biochim Biophys Acta, 1577*(2), 337-353.

Binet, J. L., Auquier, A., Dighiero, G., Chastang, C., Piguet, H., Goasguen, J., Vaugier, G., Potron, G., Colona, P., Oberling, F., Thomas, M., Tchernia, G., Jacquillat, C., Boivin, P., Lesty, C., Duault, M. T., Monconduit, M., Belabbes, S., & Gremy, F. (1981). A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer, 48*(1), 198-206.

Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A., & McCormick, F. (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science, 274*(5286), 373-376.

Black, B. L., Brewer, G., & Lyles, D. S. (1994). Effect of vesicular stomatitis virus matrix protein on host-directed translation in vivo. *J Virol, 68*(1), 555-560.

Black, B. L., & Lyles, D. S. (1992). Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo. *J Virol, 66*(7), 4058-4064.

Black, B. L., Rhodes, R. B., McKenzie, M., & Lyles, D. S. (1993). The role of vesicular stomatitis virus matrix protein in inhibition of host-directed gene expression is genetically separable from its function in virus assembly. *J Virol, 67*(8), 4814-4821.

Boldin, M. P., Mett, I. L., Varfolomeev, E. E., Chumakov, I., Shemer-Avni, Y., Camonis, J. H., & Wallach, D. (1995). Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J Biol Chem, 270*(1), 387-391.

Bonapace, L., Bornhauser, B. C., Schmitz, M., Cario, G., Ziegler, U., Niggli, F. K., Schafer, B. W., Schrappe, M., Stanulla, M., & Bourquin, J. P. (2010). Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *J Clin Invest, 120*(4), 1310-1323. doi: 10.1172/JCI39987

Boogaerts, M. A., Van Hoof, A., Catovsky, D., Kovacs, M., Montillo, M., Zinzani, P. L., Binet, J. L., Feremans, W., Marcus, R., Bosch, F., Verhoef, G., & Klein, M. (2001). Activity of oral fludarabine phosphate in previously treated chronic lymphocytic leukemia. *J Clin Oncol, 19*(22), 4252-4258.

Borner, C. (2003). The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. *Mol Immunol, 39*(11), 615-647.

Bos, J. L. (1989). ras oncogenes in human cancer: a review. *Cancer Res, 49*(17), 4682-4689.

Brenner, H., Gondos, A., & Pulte, D. (2008). Trends in long-term survival of patients with chronic lymphocytic leukemia from the 1980s to the early 21st century. *Blood, 111*(10), 4916-4921. doi: 10.1182/blood-2007-12-129379

Brooks, C., Wei, Q., Feng, L., Dong, G., Tao, Y., Mei, L., Xie, Z. J., & Dong, Z. (2007). Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. *Proc Natl Acad Sci U S A, 104*(28), 11649-11654. doi: 10.1073/pnas.0703976104

Buggins, A. G., & Pepper, C. J. (2010). The role of Bcl-2 family proteins in chronic lymphocytic leukaemia. *Leuk Res, 34*(7), 837-842. doi: 10.1016/j.leukres.2010.03.011

Bushunow, P., Reidenberg, M. M., Wasenko, J., Winfield, J., Lorenzo, B., Lemke, S., Himpler, B., Corona, R., & Coyle, T. (1999). Gossypol treatment of recurrent adult malignant gliomas. *J Neurooncol, 43*(1), 79-86.

Byrd, J. C., Kipps, T. J., Flinn, I. W., Castro, J., Lin, T. S., Wierda, W., Heerema, N., Woodworth, J., Hughes, S., Tangri, S., Harris, S., Wynne, D., Molina, A., Leigh, B., & O'Brien, S. (2010). Phase 1/2 study of lumiliximab combined with fludarabine, cyclophosphamide, and rituximab in patients with relapsed or refractory chronic lymphocytic leukemia. *Blood, 115*(3), 489-495. doi: 10.1182/blood-2009-08-237727

Cande, C., Cecconi, F., Dessen, P., & Kroemer, G. (2002). Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? *J Cell Sci, 115*(Pt 24), 4727-4734.

Cann, A. (2011). *Principles of Molecular Virology* (5th Edition ed.). Amsterdam ; London Elsevier Academic Press.

Carneiro, F. A., Bianconi, M. L., Weissmuller, G., Stauffer, F., & Da Poian, A. T. (2002). Membrane recognition by vesicular stomatitis virus involves enthalpy-driven protein-lipid interactions. *J Virol, 76*(8), 3756-3764.

Cartron, P. F., Gallenne, T., Bougras, G., Gautier, F., Manero, F., Vusio, P., Meflah, K., Vallette, F. M., & Juin, P. (2004). The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell, 16*(5), 807-818. doi: 10.1016/j.molcel.2004.10.028

Castro, J. E., Prada, C. E., Loria, O., Kamal, A., Chen, L., Burrows, F. J., & Kipps, T. J. (2005). ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia. *Blood, 106*(7), 2506-2512. doi: 10.1182/blood-2005-03-1099

Chan, A. C., Kadlecek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L., Iwashima, M., Parslow, T. G., & Weiss, A. (1994). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science, 264*(5165), 1599-1601.

Chan, D. C. (2007). Mitochondrial dynamics in disease. *N Engl J Med, 356*(17), 1707-1709. doi: 10.1056/NEJMp078040

Chao, D. T., & Korsmeyer, S. J. (1998). BCL-2 family: regulators of cell death. *Annu Rev Immunol, 16*, 395-419. doi: 10.1146/annurev.immunol.16.1.395

Chao, D. T., Linette, G. P., Boise, L. H., White, L. S., Thompson, C. B., & Korsmeyer, S. J. (1995). Bcl-XL and Bcl-2 repress a common pathway of cell death. *J Exp Med, 182*(3), 821-828.

Chen, L., Huynh, L., Apgar, J., Tang, L., Rassenti, L., Weiss, A., & Kipps, T. J. (2008). ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. *Blood, 111*(5), 2685-2692. doi: 10.1182/blood-2006-12-062265

Chen, L., Widhopf, G., Huynh, L., Rassenti, L., Rai, K. R., Weiss, A., & Kipps, T. J. (2002). Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood, 100*(13), 4609-4614. doi: 10.1182/blood-2002-06-1683

Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., Colman, P. M., Day, C. L., Adams, J. M., & Huang, D. C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell, 17*(3), 393-403. doi: 10.1016/j.molcel.2004.12.030

Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J., & Korsmeyer, S. J. (2003). VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science, 301*(5632), 513-517. doi: 10.1126/science.1083995

Chesler, D. A., Dodard, C., Lee, G. Y., Levy, D. E., & Reiss, C. S. (2004). Interferon-gamma-induced inhibition of neuronal vesicular stomatitis virus infection is STAT1 dependent. *J Neurovirol, 10*(1), 57-63.

Chesler, D. A., Munoz-Jordan, J. L., Donelan, N., Garcia-Sastre, A., & Reiss, C. S. (2003). PKR is not required for interferon-gamma inhibition of VSV replication in neurons. *Viral Immunol, 16*(1), 87-96. doi: 10.1089/088282403763635474

Chevallier, P., Penther, D., Avet-Loiseau, H., Robillard, N., Ifrah, N., Mahe, B., Hamidou, M., Maisonneuve, H., Moreau, P., Jardel, H., Harousseau, J. L., Bataille, R., & Garand, R. (2002). CD38 expression and secondary 17p deletion are important prognostic factors in chronic lymphocytic leukaemia. *Br J Haematol, 116*(1), 142-150.

Chipuk, J. E., & Green, D. R. (2009). PUMA cooperates with direct activator proteins to promote mitochondrial outer membrane permeabilization and apoptosis. *Cell Cycle, 8*(17), 2692-2696.

Choi, I. K., Lee, J. S., Zhang, S. N., Park, J., Sonn, C. H., Lee, K. M., & Yun, C. O. (2011). Oncolytic adenovirus co-expressing IL-12 and IL-18 improves tumor-specific immunity via differentiation of T cells expressing IL-12Rbeta2 or IL-18Ralpha. *Gene Ther, 18*(9), 898-909. doi: 10.1038/gt.2011.37

Choi, I. K., Lee, Y. S., Yoo, J. Y., Yoon, A. R., Kim, H., Kim, D. S., Seidler, D. G., Kim, J. H., & Yun, C. O. (2010). Effect of decorin on overcoming the extracellular matrix barrier for oncolytic virotherapy. *Gene Ther, 17*(2), 190-201. doi: 10.1038/gt.2009.142

Chou, C. H., Lee, R. S., & Yang-Yen, H. F. (2006). An internal EELD domain facilitates mitochondrial targeting of Mcl-1 via a Tom70-dependent pathway. *Mol Biol Cell, 17*(9), 3952-3963. doi: 10.1091/mbc.E06-04-0319

Chung, Y. M., Park, S. H., Tsai, W. B., Wang, S. Y., Ikeda, M. A., Berek, J. S., Chen, D. J., & Hu, M. C. (2012). FOXO3 signalling links ATM to the p53 apoptotic pathway following DNA damage. *Nat Commun, 3*, 1000. doi: 10.1038/ncomms2008

Cimmino, A., Calin, G. A., Fabbri, M., Iorio, M. V., Ferracin, M., Shimizu, M., Wojcik, S. E., Aqeilan, R. I., Zupo, S., Dono, M., Rassenti, L., Alder, H., Volinia, S., Liu, C. G., Kipps, T. J., Negrini, M., & Croce, C. M. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A, 102*(39), 13944-13949. doi: 10.1073/pnas.0506654102

Clark, S. L., Jr. (1957). Cellular differentiation in the kidneys of newborn mice studies with the electron microscope. *J Biophys Biochem Cytol, 3*(3), 349-362.

Clohessy, J. G., Zhuang, J., & Brady, H. J. (2004). Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells. *Br J Haematol, 125*(5), 655-665. doi: 10.1111/j.1365-2141.2004.04949.x

Coil, D. A., & Miller, A. D. (2004). Phosphatidylserine is not the cell surface receptor for vesicular stomatitis virus. *J Virol, 78*(20), 10920-10926. doi: 10.1128/JVI.78.20.10920-10926.2004

Comelli, M., Genero, N., & Mavelli, I. (2009). Caspase-independent apoptosis in Friend's erythroleukemia cells: role of mitochondrial ATP synthesis impairment in relocation of apoptosis-inducing factor and endonuclease G. *J Bioenerg Biomembr, 41*(1), 49-59. doi: 10.1007/s10863-009-9196-8

Cooper, G.M. (2000). *The Cell: A Molecular Approach* (2nd edition ed.). Sunderland (MA): Sinauer Associates.

Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer, 2*(9), 647-656. doi: 10.1038/nrc883

Cotter, T. G. (2009). Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer, 9*(7), 501-507. doi: 10.1038/nrc2663

Cramer, P., & Hallek, M. (2011). Prognostic factors in chronic lymphocytic leukemia-what do we need to know? *Nat Rev Clin Oncol, 8*(1), 38-47. doi: 10.1038/nrclinonc.2010.167

Critchley-Thorne, R. J., Simons, D. L., Yan, N., Miyahira, A. K., Dirbas, F. M., Johnson, D. L., Swetter, S. M., Carlson, R. W., Fisher, G. A., Koong, A., Holmes, S., & Lee, P. P. (2009). Impaired interferon signaling is a common immune defect in human cancer. *Proc Natl Acad Sci U S A, 106*(22), 9010-9015. doi: 10.1073/pnas.0901329106

Crowther-Swanepoel, D., & Houlston, R. S. (2010). Genetic variation and risk of chronic lymphocytic leukaemia. *Semin Cancer Biol, 20*(6), 363-369. doi: 10.1016/j.semcancer.2010.08.006

Cureton, D. K., Massol, R. H., Whelan, S. P., & Kirchhausen, T. (2010). The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis. *PLoS Pathog, 6*(9), e1001127. doi: 10.1371/journal.ppat.1001127

Dai, H., Smith, A., Meng, X. W., Schneider, P. A., Pang, Y. P., & Kaufmann, S. H. (2011). Transient binding of an activator BH3 domain to the Bak BH3-binding groove initiates Bak oligomerization. *J Cell Biol, 194*(1), 39-48. doi: 10.1083/jcb.201102027

Dalloul, A. (2009). CD5: a safeguard against autoimmunity and a shield for cancer cells. *Autoimmun Rev, 8*(4), 349-353. doi: 10.1016/j.autrev.2008.11.007

Danial, N. N., & Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell, 116*(2), 205-219.

Daniel, P. T., Pun, K. T., Ritschel, S., Sturm, I., Holler, J., Dorken, B., & Brown, R. (1999). Expression of the death gene Bik/Nbk promotes sensitivity to drug-induced apoptosis in corticosteroid-resistant T-cell lymphoma and prevents tumor growth in severe combined immunodeficient mice. *Blood, 94*(3), 1100-1107.

Danilov, A. V., Danilova, O. V., Klein, A. K., & Huber, B. T. (2006). Molecular pathogenesis of chronic lymphocytic leukemia. *Curr Mol Med, 6*(6), 665-675.

Davids, M. S., & Letai, A. (2012). Targeting the B-cell lymphoma/leukemia 2 family in cancer. *J Clin Oncol, 30*(25), 3127-3135. doi: 10.1200/JCO.2011.37.0981

Davison, A. J., Benko, M., & Harrach, B. (2003). Genetic content and evolution of adenoviruses. *J Gen Virol, 84*(Pt 11), 2895-2908.

Day, C. L., Chen, L., Richardson, S. J., Harrison, P. J., Huang, D. C., & Hinds, M. G. (2005). Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. *J Biol Chem, 280*(6), 4738-4744. doi: 10.1074/jbc.M411434200

de Weerd, N. A., & Nguyen, T. (2012). The interferons and their receptors--distribution and regulation. *Immunol Cell Biol, 90*(5), 483-491. doi: 10.1038/icb.2012.9

Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y., Nelson, D. A., Jin, S., & White, E. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell, 10*(1), 51-64. doi: 10.1016/j.ccr.2006.06.001

Dejean, L. M., Martinez-Caballero, S., & Kinnally, K. W. (2006). Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis? *Cell Death Differ, 13*(8), 1387-1395. doi: 10.1038/sj.cdd.4401949

Del Gaizo Moore, V., Brown, J. R., Certo, M., Love, T. M., Novina, C. D., & Letai, A. (2007). Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest, 117*(1), 112-121. doi: 10.1172/JCI28281

Del Gaizo Moore, V., Schlis, K. D., Sallan, S. E., Armstrong, S. A., & Letai, A. (2008). BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. *Blood, 111*(4), 2300-2309. doi: 10.1182/blood-2007-06-098012

DeLong, M. J. (1998). Apoptosis: a modulator of cellular homeostasis and disease states. *Ann N Y Acad Sci, 842*, 82-90.

Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., & Amiot, M. (2002). Antisense strategy shows that Mcl-1 rather

than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood, 100*(1), 194-199.

Deter, R. L., & De Duve, C. (1967). Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. *J Cell Biol, 33*(2), 437-449.

Dewson, G., Kratina, T., Sim, H. W., Puthalakath, H., Adams, J. M., Colman, P. M., & Kluck, R. M. (2008). To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. *Mol Cell, 30*(3), 369-380. doi: 10.1016/j.molcel.2008.04.005

Ding, Q., He, X., Hsu, J. M., Xia, W., Chen, C. T., Li, L. Y., Lee, D. F., Liu, J. C., Zhong, Q., Wang, X., & Hung, M. C. (2007). Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Mol Cell Biol, 27*(11), 4006-4017. doi: 10.1128/MCB.00620-06

Dohner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Krober, A., Bullinger, L., Dohner, K., Bentz, M., & Lichter, P. (2000). Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med, 343*(26), 1910-1916. doi: 10.1056/NEJM200012283432602

Dohner, H., Stilgenbauer, S., James, M. R., Benner, A., Weilguni, T., Bentz, M., Fischer, K., Hunstein, W., & Lichter, P. (1997). 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. *Blood, 89*(7), 2516-2522.

Dorig, R. E., Marcil, A., Chopra, A., & Richardson, C. D. (1993). The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell, 75*(2), 295-305.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer, 3*(1), 11-22. doi: 10.1038/nrc969

Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A., & Parslow, T. G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science, 264*(5165), 1596-1599.

Elgendy, M., Sheridan, C., Brumatti, G., & Martin, S. J. (2011). Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. *Mol Cell, 42*(1), 23-35. doi: 10.1016/j.molcel.2011.02.009

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi: 10.1080/01926230701320337

Errington, F., Steele, L., Prestwich, R., Harrington, K. J., Pandha, H. S., Vidal, L., de Bono, J., Selby, P., Coffey, M., Vile, R., & Melcher, A. (2008). Reovirus activates human dendritic cells to promote innate antitumor immunity. *J Immunol, 180*(9), 6018-6026.

Errington, F., White, C. L., Twigger, K. R., Rose, A., Scott, K., Steele, L., Ilett, L. J., Prestwich, R., Pandha, H. S., Coffey, M., Selby, P., Vile, R., Harrington, K. J., & Melcher, A. A. (2008). Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. *Gene Ther, 15*(18), 1257-1270. doi: 10.1038/gt.2008.58

Eskelinen, E. L., Tanaka, Y., & Saftig, P. (2003). At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol, 13*(3), 137-145.

Etoh, T., Himeno, Y., Matsumoto, T., Aramaki, M., Kawano, K., Nishizono, A., & Kitano, S. (2003). Oncolytic viral therapy for human pancreatic cancer cells by reovirus. *Clin Cancer Res, 9*(3), 1218-1223.

Fader, C. M., Sanchez, D. G., Mestre, M. B., & Colombo, M. I. (2009). TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. *Biochim Biophys Acta, 1793*(12), 1901-1916. doi: 10.1016/j.bbamcr.2009.09.011

Faria, P. A., Chakraborty, P., Levay, A., Barber, G. N., Ezelle, H. J., Enninga, J., Arana, C., van Deursen, J., & Fontoura, B. M. (2005). VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway. *Mol Cell, 17*(1), 93-102. doi: 10.1016/j.molcel.2004.11.023

Fedorov, L. M., Schmittwolf, C., Amann, K., Thomas, W. H., Muller, A. M., Schubert, H., Domen, J., & Kneitz, B. (2006). Renal failure causes early death of bcl-2 deficient mice. *Mech Ageing Dev, 127*(7), 600-609. doi: 10.1016/j.mad.2006.02.009

Ferran, M. C., & Lucas-Lenard, J. M. (1997). The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. *J Virol, 71*(1), 371-377.

Fishelson, Z., Donin, N., Zell, S., Schultz, S., & Kirschfink, M. (2003). Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. *Mol Immunol, 40*(2-4), 109-123.

Frey, T. G., & Mannella, C. A. (2000). The internal structure of mitochondria. *Trends Biochem Sci, 25*(7), 319-324.

Fulda, S. (2008). Targeting inhibitor of apoptosis proteins (IAPs) for cancer therapy. *Anticancer Agents Med Chem, 8*(5), 533-539.

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: cell survival and cell death. *Int J Cell Biol, 2010*, 214074. doi: 10.1155/2010/214074

Funaro, A., Spagnoli, G. C., Ausiello, C. M., Alessio, M., Roggero, S., Delia, D., Zaccolo, M., & Malavasi, F. (1990). Involvement of the multilineage CD38 molecule in a unique pathway of cell activation and proliferation. *J Immunol, 145*(8), 2390-2396.

Galanis, E., Bateman, A., Johnson, K., Diaz, R. M., James, C. D., Vile, R., & Russell, S. J. (2001). Use of viral fusogenic membrane glycoproteins as novel therapeutic transgenes in gliomas. *Hum Gene Ther, 12*(7), 811-821. doi: 10.1089/104303401750148766

Gale, R. P., Cozen, W., Goodman, M. T., Wang, F. F., & Bernstein, L. (2000). Decreased chronic lymphocytic leukemia incidence in Asians in Los Angeles County. *Leuk Res, 24*(8), 665-669.

Gandhi, L., Camidge, D. R., Ribeiro de Oliveira, M., Bonomi, P., Gandara, D., Khaira, D., Hann, C. L., McKeegan, E. M., Litvinovich, E., Hemken, P. M., Dive, C., Enschede, S. H., Nolan, C., Chiu, Y. L., Busman, T., Xiong, H., Krivoshik, A. P., Humerickhouse, R., Shapiro, G. I., & Rudin, C. M. (2011).

Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. *J Clin Oncol, 29*(7), 909-916. doi: 10.1200/JCO.2010.31.6208

Ganley, I. G., Lam du, H., Wang, J., Ding, X., Chen, S., & Jiang, X. (2009). ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem, 284*(18), 12297-12305. doi: 10.1074/jbc.M900573200

Gannage, M., & Munz, C. (2009). Autophagy in MHC class II presentation of endogenous antigens. *Curr Top Microbiol Immunol, 335*, 123-140. doi: 10.1007/978-3-642-00302-8_6

Garcia-Munoz, R., Galiacho, V. R., & Llorente, L. (2012). Immunological aspects in chronic lymphocytic leukemia (CLL) development. *Ann Hematol, 91*(7), 981-996. doi: 10.1007/s00277-012-1460-z

Gentile, M., Cutrona, G., Neri, A., Molica, S., Ferrarini, M., & Morabito, F. (2009). Predictive value of beta2-microglobulin (beta2-m) levels in chronic lymphocytic leukemia since Binet A stages. *Haematologica, 94*(6), 887-888. doi: 10.3324/haematol.2009.005561

George, N. M., Evans, J. J., & Luo, X. (2007). A three-helix homo-oligomerization domain containing BH3 and BH1 is responsible for the apoptotic activity of Bax. *Genes Dev, 21*(15), 1937-1948. doi: 10.1101/gad.1553607

Gerber, N. N. (1975). Prodigiosin-like pigments. *CRC Crit Rev Microbiol, 3*(4), 469-485.

Germain, M., & Duronio, V. (2007). The N terminus of the anti-apoptotic BCL-2 homologue MCL-1 regulates its localization and function. *J Biol Chem, 282*(44), 32233-32242. doi: 10.1074/jbc.M706408200

Germain, M., & Shore, G. C. (2003). Cellular distribution of Bcl-2 family proteins. *Sci STKE, 2003*(173), pe10. doi: 10.1126/stke.2003.173.pe10

Gladstone, D. E., & Fuchs, E. (2012). Hematopoietic stem cell transplantation for chronic lymphocytic leukemia. *Curr Opin Oncol, 24*(2), 176-181. doi: 10.1097/CCO.0b013e32834f8011

Gobessi, S., Laurenti, L., Longo, P. G., Sica, S., Leone, G., & Efremov, D. G. (2007). ZAP-70 enhances B-cell-receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. *Blood, 109*(5), 2032-2039. doi: 10.1182/blood-2006-03-011759

Goldin, L. R., Bjorkholm, M., Kristinsson, S. Y., Turesson, I., & Landgren, O. (2009). Elevated risk of chronic lymphocytic leukemia and other indolent non-Hodgkin's lymphomas among relatives of patients with chronic lymphocytic leukemia. *Haematologica, 94*(5), 647-653. doi: 10.3324/haematol.2008.003632

Gonzalvez, F., & Ashkenazi, A. (2010). New insights into apoptosis signaling by Apo2L/TRAIL. *Oncogene, 29*(34), 4752-4765. doi: 10.1038/onc.2010.221

Gordy, C., & He, Y. W. (2012). The crosstalk between autophagy and apoptosis: where does this lead? *Protein Cell, 3*(1), 17-27. doi: 10.1007/s13238-011-1127-x

Goubau, D., Romieu-Mourez, R., Solis, M., Hernandez, E., Mesplede, T., Lin, R., Leaman, D., & Hiscott, J. (2009). Transcriptional re-programming of primary macrophages reveals distinct apoptotic and anti-tumoral functions of IRF-3 and IRF-7. *Eur J Immunol, 39*(2), 527-540. doi: 10.1002/eji.200838832

Gozzetti, A., Crupi, R., & Tozzuoli, D. (2004). The use of fluorescence in situ hybridization (FISH) in chronic lymphocytic leukemia (CLL). *Hematology, 9*(1), 11-15. doi: 10.1080/10245330310001652446

Gray, C. W., Ward, R. V., Karran, E., Turconi, S., Rowles, A., Viglienghi, D., Southan, C., Barton, A., Fantom, K. G., West, A., Savopoulos, J., Hassan, N. J., Clinkenbeard, H., Hanning, C., Amegadzie, B., Davis, J. B., Dingwall, C., Livi, G. P., & Creasy, C. L. (2000). Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. *Eur J Biochem, 267*(18), 5699-5710.

Gribben, J. G. (2010). How I treat CLL up front. *Blood, 115*(2), 187-197. doi: 10.1182/blood-2009-08-207126

Griffin, D.E. (2007). *Fields Virology* (H. P. Knipe DM, Griffin DE, et al Ed. 5Th Edition ed.). Philadelphia, PA: Lippincott Williams & Wilkins.

Grote, D., Russell, S. J., Cornu, T. I., Cattaneo, R., Vile, R., Poland, G. A., & Fielding, A. K. (2001). Live attenuated measles virus induces regression of human lymphoma xenografts in immunodeficient mice. *Blood, 97*(12), 3746-3754.

Guo, J. Y., Chen, H. Y., Mathew, R., Fan, J., Strohecker, A. M., Karsli-Uzunbas, G., Kamphorst, J. J., Chen, G., Lemons, J. M., Karantza, V., Coller, H. A., Dipaola, R. S., Gelinas, C., Rabinowitz, J. D., & White, E. (2011). Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev, 25*(5), 460-470. doi: 10.1101/gad.2016311

Haenszel, W., & Kurihara, M. (1968). Studies of Japanese migrants. I. Mortality from cancer and other diseases among Japanese in the United States. *J Natl Cancer Inst, 40*(1), 43-68.

Hamblin, A. D., & Hamblin, T. J. (2005). Functional and prognostic role of ZAP-70 in chronic lymphocytic leukaemia. *Expert Opin Ther Targets, 9*(6), 1165-1178. doi: 10.1517/14728222.9.6.1165

Hamblin, T. J., Davis, Z., Gardiner, A., Oscier, D. G., & Stevenson, F. K. (1999). Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood, 94*(6), 1848-1854.

Hamblin, T. J., Orchard, J. A., Gardiner, A., Oscier, D. G., Davis, Z., & Stevenson, F. K. (2000). Immunoglobulin V genes and CD38 expression in CLL. *Blood, 95*(7), 2455-2457.

Hamblin, T. J., Orchard, J. A., Ibbotson, R. E., Davis, Z., Thomas, P. W., Stevenson, F. K., & Oscier, D. G. (2002). CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. *Blood, 99*(3), 1023-1029.

Hanada, M., Delia, D., Aiello, A., Stadtmauer, E., & Reed, J. C. (1993). bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood, 82*(6), 1820-1828.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Happo, L., Strasser, A., & Cory, S. (2012). BH3-only proteins in apoptosis at a glance. *J Cell Sci, 125*(Pt 5), 1081-1087. doi: 10.1242/jcs.090514

Hayat, A., O'Brien, D., O'Rourke, P., McGuckin, S., Fitzgerald, T., Conneally, E., Browne, P. V., McCann, S. R., Lawler, M. P., & Vandenberghe, E. (2006). CD38 expression level and pattern of expression remains a reliable and robust marker of progressive disease in chronic lymphocytic leukemia. *Leuk Lymphoma, 47*(11), 2371-2379. doi: 10.1080/10428190600947727

He, H., Dang, Y., Dai, F., Guo, Z., Wu, J., She, X., Pei, Y., Chen, Y., Ling, W., Wu, C., Zhao, S., Liu, J. O., & Yu, L. (2003). Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B. *J Biol Chem, 278*(31), 29278-29287. doi: 10.1074/jbc.M303800200

Hemann, M. T., Zilfou, J. T., Zhao, Z., Burgess, D. J., Hannon, G. J., & Lowe, S. W. (2004). Suppression of tumorigenesis by the p53 target PUMA. *Proc Natl Acad Sci U S A, 101*(25), 9333-9338. doi: 10.1073/pnas.0403286101

Hernandez, J. M., Mecucci, C., Criel, A., Meeus, P., Michaux, I., Van Hoof, A., Verhoef, G., Louwagie, A., Scheiff, J. M., Michaux, J. L., & et al. (1995). Cytogenetic analysis of B cell chronic lymphoid leukemias classified according to morphologic and immunophenotypic (FAB) criteria. *Leukemia, 9*(12), 2140-2146.

Hinds, M. G., Lackmann, M., Skea, G. L., Harrison, P. J., Huang, D. C., & Day, C. L. (2003). The structure of Bcl-w reveals a role for the C-terminal residues in modulating biological activity. *EMBO J, 22*(7), 1497-1507. doi: 10.1093/emboj/cdg144

Hirasawa, K., Nishikawa, S. G., Norman, K. L., Alain, T., Kossakowska, A., & Lee, P. W. (2002). Oncolytic reovirus against ovarian and colon cancer. *Cancer Res, 62*(6), 1696-1701.

Hochman, A., Liang, H., Offen, D., Melamed, E., & Sternin, H. (2000). Developmental changes in antioxidant enzymes and oxidative damage in kidneys, liver and brain of bcl-2 knockout mice. *Cell Mol Biol (Noisy-le-grand), 46*(1), 41-52.

Hockenbery, D. M. (2010). Targeting mitochondria for cancer therapy. *Environ Mol Mutagen, 51*(5), 476-489. doi: 10.1002/em.20552

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., & Korsmeyer, S. J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature, 348*(6299), 334-336. doi: 10.1038/348334a0

Hollstein, M., Sidransky, D., Vogelstein, B., & Harris, C. C. (1991). p53 mutations in human cancers. *Science, 253*(5015), 49-53.

Hosokawa, N., Sasaki, T., Iemura, S., Natsume, T., Hara, T., & Mizushima, N. (2009). Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy, 5*(7), 973-979.

Houlston, R. S., & Catovsky, D. (2008). Familial chronic lymphocytic leukemia. *Curr Hematol Malig Rep, 3*(4), 221-225. doi: 10.1007/s11899-008-0031-9

Houlston, R. S., Sellick, G., Yuille, M., Matutes, E., & Catovsky, D. (2003). Causation of chronic lymphocytic leukemia--insights from familial disease. *Leuk Res, 27*(10), 871-876.

Huang, Q., Petros, A. M., Virgin, H. W., Fesik, S. W., & Olejniczak, E. T. (2002). Solution structure of a Bcl-2 homolog from Kaposi sarcoma virus. *Proc Natl Acad Sci U S A, 99*(6), 3428-3433. doi: 10.1073/pnas.062525799

Hussain, S. R., Cheney, C. M., Johnson, A. J., Lin, T. S., Grever, M. R., Caligiuri, M. A., Lucas, D. M., & Byrd, J. C. (2007). Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity. *Clin Cancer Res, 13*(7), 2144-2150. doi: 10.1158/1078-0432.CCR-06-2294

Hwang, J. J., Kuruvilla, J., Mendelson, D., Pishvaian, M. J., Deeken, J. F., Siu, L. L., Berger, M. S., Viallet, J., & Marshall, J. L. (2010). Phase I dose finding studies of obatoclax (GX15-070), a small molecule pan-BCL-2 family antagonist, in patients with advanced solid tumors or lymphoma. *Clin Cancer Res, 16*(15), 4038-4045. doi: 10.1158/1078-0432.CCR-10-0822

Iankov, I. D., Msaouel, P., Allen, C., Federspiel, M. J., Bulur, P. A., Dietz, A. B., Gastineau, D., Ikeda, Y., Ingle, J. N., Russell, S. J., & Galanis, E. (2010). Demonstration of anti-tumor activity of oncolytic measles virus strains in a malignant pleural effusion breast cancer model. *Breast Cancer Res Treat, 122*(3), 745-754. doi: 10.1007/s10549-009-0602-z

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer, 2*(4), 277-288. doi: 10.1038/nrc776

Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Merry, A., Chen, S., & Nunez, G. (1998). Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-XL. *J Biol Chem, 273*(15), 8705-8710.

Jackson, G. G., & Muldoon, R. L. (1973). Viruses causing common respiratory infection in man. IV. Reoviruses and Adenoviruses. *J Infect Dis, 128*(6), 811-866.

Jackson, W. T., Giddings, T. H., Jr., Taylor, M. P., Mulinyawe, S., Rabinovitch, M., Kopito, R. R., & Kirkegaard, K. (2005). Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol, 3*(5), e156. doi: 10.1371/journal.pbio.0030156

Jager, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., & Eskelinen, E. L. (2004). Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci, 117*(Pt 20), 4837-4848. doi: 10.1242/jcs.01370

Janeway CA Jr, Travers P, Walport M, et al. (2001). *Immunobiology: The Immune System in Health and Disease.* (5th Edition ed.). New York: Garland Science.

Jarskog, L. F., Gilmore, J. H., Selinger, E. S., & Lieberman, J. A. (2000). Cortical bcl-2 protein expression and apoptotic regulation in schizophrenia. *Biol Psychiatry, 48*(7), 641-650.

Jelinek, D. F., Tschumper, R. C., Geyer, S. M., Bone, N. D., Dewald, G. W., Hanson, C. A., Stenson, M. J., Witzig, T. E., Tefferi, A., & Kay, N. E. (2001). Analysis of clonal B-cell CD38 and immunoglobulin variable region

sequence status in relation to clinical outcome for B-chronic lymphocytic leukaemia. *Br J Haematol, 115*(4), 854-861.

Johnson, K. M., Vogel, J. E., & Peralta, P. H. (1966). Clinical and serological response to laboratory-acquired human infection by Indiana type vesicular stomatitis virus (VSV). *Am J Trop Med Hyg, 15*(2), 244-246.

Jounai, N., Takeshita, F., Kobiyama, K., Sawano, A., Miyawaki, A., Xin, K. Q., Ishii, K. J., Kawai, T., Akira, S., Suzuki, K., & Okuda, K. (2007). The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc Natl Acad Sci U S A, 104*(35), 14050-14055. doi: 10.1073/pnas.0704014104

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., & Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J, 19*(21), 5720-5728. doi: 10.1093/emboj/19.21.5720

Kang, R., Zeh, H. J., Lotze, M. T., & Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ, 18*(4), 571-580. doi: 10.1038/cdd.2010.191

Kaufmann, T., Schinzel, A., & Borner, C. (2004). Bcl-w(edding) with mitochondria. *Trends Cell Biol, 14*(1), 8-12.

Kaufmann, T., Tai, L., Ekert, P. G., Huang, D. C., Norris, F., Lindemann, R. K., Johnstone, R. W., Dixit, V. M., & Strasser, A. (2007). The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell, 129*(2), 423-433. doi: 10.1016/j.cell.2007.03.017

Ke, F., Voss, A., Kerr, J. B., O'Reilly, L. A., Tai, L., Echeverry, N., Bouillet, P., Strasser, A., & Kaufmann, T. (2012). BCL-2 family member BOK is widely expressed but its loss has only minimal impact in mice. *Cell Death Differ, 19*(6), 915-925. doi: 10.1038/cdd.2011.210

Kelly, E., & Russell, S. J. (2007). History of oncolytic viruses: genesis to genetic engineering. *Mol Ther, 15*(4), 651-659. doi: 10.1038/sj.mt.6300108

Kelly, K., Nawrocki, S., Mita, A., Coffey, M., Giles, F. J., & Mita, M. (2009). Reovirus-based therapy for cancer. *Expert Opin Biol Ther, 9*(7), 817-830. doi: 10.1517/14712590903002039

Kelly, P. N., & Strasser, A. (2011). The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ, 18*(9), 1414-1424. doi: 10.1038/cdd.2011.17

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer, 26*(4), 239-257.

Khuri, F. R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I. F., Romel, L., Gore, M., Ironside, J., MacDougall, R. H., Heise, C., Randlev, B., Gillenwater, A. M., Bruso, P., Kaye, S. B., Hong, W. K., & Kirn, D. H. (2000). a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med, 6*(8), 879-885. doi: 10.1038/78638

Kilani, R. T., Tamimi, Y., Hanel, E. G., Wong, K. K., Karmali, S., Lee, P. W., & Moore, R. B. (2003). Selective reovirus killing of bladder cancer in a co-culture spheroid model. *Virus Res, 93*(1), 1-12.

Kim, E., Kim, J. H., Shin, H. Y., Lee, H., Yang, J. M., Kim, J., Sohn, J. H., Kim, H., & Yun, C. O. (2003). Ad-mTERT-delta19, a conditional replication-competent adenovirus driven by the human telomerase promoter, selectively replicates in and elicits cytopathic effect in a cancer cell-specific manner. *Hum Gene Ther, 14*(15), 1415-1428. doi: 10.1089/104303403769211637

Kim, J. H., Lee, Y. S., Kim, H., Huang, J. H., Yoon, A. R., & Yun, C. O. (2006). Relaxin expression from tumor-targeting adenoviruses and its intratumoral spread, apoptosis induction, and efficacy. *J Natl Cancer Inst, 98*(20), 1482-1493. doi: 10.1093/jnci/djj397

Kim, J., Huang, W. P., Stromhaug, P. E., & Klionsky, D. J. (2002). Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. *J Biol Chem, 277*(1), 763-773. doi: 10.1074/jbc.M109134200

Klionsky, D. J., Cuervo, A. M., Dunn, W. A., Jr., Levine, B., van der Klei, I., & Seglen, P. O. (2007). How shall I eat thee? *Autophagy, 3*(5), 413-416.

Knauf, W. U., Langenmayer, I., Ehlers, B., Mohr, B., Adorf, D., Nerl, C. H., Hallek, M., Zwingers, T. H., Emmerich, B., & Thiel, E. (1997). Serum levels of soluble CD23, but not soluble CD25, predict disease progression in early stage B-cell chronic lymphocytic leukemia. *Leuk Lymphoma, 27*(5-6), 523-532. doi: 10.3109/10428199709058320

Knauf, W. U., Lissichkov, T., Aldaoud, A., Liberati, A., Loscertales, J., Herbrecht, R., Juliusson, G., Postner, G., Gercheva, L., Goranov, S., Becker, M., Fricke, H. J., Huguet, F., Del Giudice, I., Klein, P., Tremmel, L., Merkle, K., & Montillo, M. (2009). Phase III randomized study of bendamustine compared with chlorambucil in previously untreated patients with chronic lymphocytic leukemia. *J Clin Oncol, 27*(26), 4378-4384. doi: 10.1200/JCO.2008.20.8389

Kobayashi, S., Lee, S. H., Meng, X. W., Mott, J. L., Bronk, S. F., Werneburg, N. W., Craig, R. W., Kaufmann, S. H., & Gores, G. J. (2007). Serine 64 phosphorylation enhances the antiapoptotic function of Mcl-1. *J Biol Chem, 282*(25), 18407-18417. doi: 10.1074/jbc.M610010200

Komatsu, T., & Reiss, C. S. (1997). IFN-gamma is not required in the IL-12 response to vesicular stomatitis virus infection of the olfactory bulb. *J Immunol, 159*(7), 3444-3452.

Kondo, K., Obitsu, S., Ohta, S., Matsunami, K., Otsuka, H., & Teshima, R. (2010). Poly(ADP-ribose) polymerase (PARP)-1-independent apoptosis-inducing factor (AIF) release and cell death are induced by eleostearic acid and blocked by alpha-tocopherol and MEK inhibition. *J Biol Chem, 285*(17), 13079-13091. doi: 10.1074/jbc.M109.044206

Konopleva, M., Contractor, R., Tsao, T., Samudio, I., Ruvolo, P. P., Kitada, S., Deng, X., Zhai, D., Shi, Y. X., Sneed, T., Verhaegen, M., Soengas, M., Ruvolo, V. R., McQueen, T., Schober, W. D., Watt, J. C., Jiffar, T., Ling, X., Marini, F. C., Harris, D., Dietrich, M., Estrov, Z., McCubrey, J., May, W. S., Reed, J. C., & Andreeff, M. (2006). Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell, 10*(5), 375-388. doi: 10.1016/j.ccr.2006.10.006

Konopleva, M., Watt, J., Contractor, R., Tsao, T., Harris, D., Estrov, Z., Bornmann, W., Kantarjian, H., Viallet, J., Samudio, I., & Andreeff, M. (2008). Mechanisms of antileukemic activity of the novel Bcl-2 homology domain-3 mimetic GX15-070 (obatoclax). *Cancer Res, 68*(9), 3413-3420. doi: 10.1158/0008-5472.CAN-07-1919

Kozmik, Z., Wang, S., Dorfler, P., Adams, B., & Busslinger, M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol Cell Biol, 12*(6), 2662-2672.

Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., & Craig, R. W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci U S A, 90*(8), 3516-3520.

Krammer, P. H. (2000). CD95's deadly mission in the immune system. *Nature, 407*(6805), 789-795. doi: 10.1038/35037728

Krober, A., Bloehdorn, J., Hafner, S., Buhler, A., Seiler, T., Kienle, D., Winkler, D., Bangerter, M., Schlenk, R. F., Benner, A., Lichter, P., Dohner, H., & Stilgenbauer, S. (2006). Additional genetic high-risk features such as 11q deletion, 17p deletion, and V3-21 usage characterize discordance of ZAP-70 and VH mutation status in chronic lymphocytic leukemia. *J Clin Oncol, 24*(6), 969-975. doi: 10.1200/JCO.2005.03.7184

Kroemer, G., El-Deiry, W. S., Golstein, P., Peter, M. E., Vaux, D., Vandenabeele, P., Zhivotovsky, B., Blagosklonny, M. V., Malorni, W., Knight, R. A., Piacentini, M., Nagata, S., Melino, G., & Nomenclature Committee on Cell, Death. (2005). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ, 12 Suppl 2*, 1463-1467. doi: 10.1038/sj.cdd.4401724

Kulik, G., & Weber, M. J. (1998). Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. *Mol Cell Biol, 18*(11), 6711-6718.

Kumar V, Abbas AK, Fausto N, Aster JC. (2010). Robins and Cotran: pathologic basis of disease (Vol. 8, pp. 25-32). Philadelphia: Saunders Elsevier.

Kurilla, M. G., Piwnica-Worms, H., & Keene, J. D. (1982). Rapid and transient localization of the leader RNA of vesicular stomatitis virus in the nuclei of infected cells. *Proc Natl Acad Sci U S A, 79*(17), 5240-5244.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., & Newmeyer, D. D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell, 17*(4), 525-535. doi: 10.1016/j.molcel.2005.02.003

Kwon, O. J., Kim, P. H., Huyn, S., Wu, L., Kim, M., & Yun, C. O. (2010). A hypoxia- and {alpha}-fetoprotein-dependent oncolytic adenovirus exhibits specific killing of hepatocellular carcinomas. *Clin Cancer Res, 16*(24), 6071-6082. doi: 10.1158/1078-0432.CCR-10-0664

Lavrik, I. N., Golks, A., & Krammer, P. H. (2005). Caspases: pharmacological manipulation of cell death. *J Clin Invest, 115*(10), 2665-2672. doi: 10.1172/JCI26252

Lee, A. S., Burdeinick-Kerr, R., & Whelan, S. P. (2013). A ribosome-specialized translation initiation pathway is required for cap-dependent translation of vesicular stomatitis virus mRNAs. *Proc Natl Acad Sci U S A, 110*(1), 324-329. doi: 10.1073/pnas.1216454109

Lee, H. K., Lund, J. M., Ramanathan, B., Mizushima, N., & Iwasaki, A. (2007). Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science, 315*(5817), 1398-1401. doi: 10.1126/science.1136880

Lee, Y. S., Kim, J. H., Choi, K. J., Choi, I. K., Kim, H., Cho, S., Cho, B. C., & Yun, C. O. (2006). Enhanced antitumor effect of oncolytic adenovirus expressing interleukin-12 and B7-1 in an immunocompetent murine model. *Clin Cancer Res, 12*(19), 5859-5868. doi: 10.1158/1078-0432.CCR-06-0935

Lemery, S. J., Zhang, J., Rothmann, M. D., Yang, J., Earp, J., Zhao, H., McDougal, A., Pilaro, A., Chiang, R., Gootenberg, J. E., Keegan, P., & Pazdur, R. (2010). U.S. Food and Drug Administration approval: ofatumumab for the treatment of patients with chronic lymphocytic leukemia refractory to fludarabine and alemtuzumab. *Clin Cancer Res, 16*(17), 4331-4338. doi: 10.1158/1078-0432.CCR-10-0570

Leuenroth, S. J., Grutkoski, P. S., Ayala, A., & Simms, H. H. (2000). The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis. *J Leukoc Biol, 68*(1), 158-166.

Levine, B., & Deretic, V. (2007). Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol, 7*(10), 767-777. doi: 10.1038/nri2161

Levine, B., & Klionsky, D. J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell, 6*(4), 463-477.

Levine, B., Mizushima, N., & Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature, 469*(7330), 323-335. doi: 10.1038/nature09782

Li, H., Zhu, H., Xu, C. J., & Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell, 94*(4), 491-501.

Li, J., Lee, B., & Lee, A. S. (2006). Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *J Biol Chem, 281*(11), 7260-7270. doi: 10.1074/jbc.M509868200

Li, J., Viallet, J., & Haura, E. B. (2008). A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. *Cancer Chemother Pharmacol, 61*(3), 525-534. doi: 10.1007/s00280-007-0499-3

Li, L. Y., Luo, X., & Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature, 412*(6842), 95-99. doi: 10.1038/35083620

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., & Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell, 91*(4), 479-489.

Lian, J., Karnak, D., & Xu, L. (2010). The Bcl-2-Beclin 1 interaction in (-)-gossypol-induced autophagy versus apoptosis in prostate cancer cells. *Autophagy, 6*(8), 1201-1203. doi: 10.1038/cdd.2010.74

Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B. H., & Jung, J. U. (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol, 8*(7), 688-699. doi: 10.1038/ncb1426

Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., & Levine, B. (1998). Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol, 72*(11), 8586-8596.

Lichtman, M. A. (2008). Battling the hematological malignancies: the 200 years' war. *Oncologist, 13*(2), 126-138. doi: 10.1634/theoncologist.2007-0228

Lichty, B. D., Power, A. T., Stojdl, D. F., & Bell, J. C. (2004). Vesicular stomatitis virus: re-inventing the bullet. *Trends Mol Med, 10*(5), 210-216. doi: 10.1016/j.molmed.2004.03.003

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell, 86*(1), 147-157.

Lloyd, J. B. (1996). Metabolite efflux and influx across the lysosome membrane. *Subcell Biochem, 27*, 361-386.

Lockshin, R. A., & Zakeri, Z. (2004). Caspase-independent cell death? *Oncogene, 23*(16), 2766-2773. doi: 10.1038/sj.onc.1207514

Lodish H, Berk A, Zipursky SL, et al. (2000). *Molecular Cell Biology* (W. H. Freeman Ed. 4th edition ed.). New York.

Long, J. S., & Ryan, K. M. (2012). New frontiers in promoting tumour cell death: targeting apoptosis, necroptosis and autophagy. *Oncogene, 31*(49), 5045-5060. doi: 10.1038/onc.2012.7

Lu, Y., Lemon, W., Liu, P. Y., Yi, Y., Morrison, C., Yang, P., Sun, Z., Szoke, J., Gerald, W. L., Watson, M., Govindan, R., & You, M. (2006). A gene expression signature predicts survival of patients with stage I non-small cell lung cancer. *PLoS Med, 3*(12), e467. doi: 10.1371/journal.pmed.0030467

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., & Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell, 94*(4), 481-490.

Lyles, D. S. (2000). Cytopathogenesis and inhibition of host gene expression by RNA viruses. *Microbiol Mol Biol Rev, 64*(4), 709-724.

Lyles, D. S. & Rupprecht, C. E. (2007). Rhabdoviridae *Fields Virology* (5th Edition ed., pp. 1363–1408). Philadelphia, PA:: Lippincott Williams & Wilkins.

Ma, X., Jin, M., Cai, Y., Xia, H., Long, K., Liu, J., Yu, Q., & Yuan, J. (2011). Mitochondrial electron transport chain complex III is required for antimycin A to inhibit autophagy. *Chem Biol, 18*(11), 1474-1481. doi: 10.1016/j.chembiol.2011.08.009

Macoska, J. A., Adsule, S., Tantivejkul, K., Wang, S., Pienta, K. J., & Lee, C. T. (2008). -(-)Gossypol promotes the apoptosis of bladder cancer cells in vitro. *Pharmacol Res, 58*(5-6), 323-331. doi: 10.1016/j.phrs.2008.09.005

MacVicar GR, Kuzel TM, Curti BD. (2008). An open-label, multicenter, phase I/II study of AT-101 in combination with docetaxel (D) and prednisone (P) in men with hormone refractory prostate cancer (HRPC). *Journal of Clinical Oncology 26*.

Maiuri, M. C., Le Toumelin, G., Criollo, A., Rain, J. C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N., Hickman, J. A., Geneste, O., & Kroemer, G. (2007). Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO J, 26*(10), 2527-2539. doi: 10.1038/sj.emboj.7601689

Malavasi, F., Deaglio, S., Damle, R., Cutrona, G., Ferrarini, M., & Chiorazzi, N. (2011). CD38 and chronic lymphocytic leukemia: a decade later. *Blood, 118*(13), 3470-3478. doi: 10.1182/blood-2011-06-275610

Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A. L., Ortolan, E., Vaisitti, T., & Aydin, S. (2008). Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev, 88*(3), 841-886. doi: 10.1152/physrev.00035.2007

Manero, F., Gautier, F., Gallenne, T., Cauquil, N., Gree, D., Cartron, P. F., Geneste, O., Gree, R., Vallette, F. M., & Juin, P. (2006). The small organic compound HA14-1 prevents Bcl-2 interaction with Bax to sensitize malignant glioma cells to induction of cell death. *Cancer Res, 66*(5), 2757-2764. doi: 10.1158/0008-5472.CAN-05-2097

Mapara, M. Y., & Sykes, M. (2004). Tolerance and cancer: mechanisms of tumor evasion and strategies for breaking tolerance. *J Clin Oncol, 22*(6), 1136-1151. doi: 10.1200/JCO.2004.10.041

Masliah, E., Mallory, M., Alford, M., Tanaka, S., & Hansen, L. A. (1998). Caspase dependent DNA fragmentation might be associated with excitotoxicity in Alzheimer disease. *J Neuropathol Exp Neurol, 57*(11), 1041-1052.

Matsunaga, K., Saitoh, T., Tabata, K., Omori, H., Satoh, T., Kurotori, N., Maejima, I., Shirahama-Noda, K., Ichimura, T., Isobe, T., Akira, S., Noda, T., & Yoshimori, T. (2009). Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol, 11*(4), 385-396. doi: 10.1038/ncb1846

Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E., & Green, D. R. (2006). Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell, 21*(6), 749-760. doi: 10.1016/j.molcel.2006.02.009

Mazumder, S., Choudhary, G. S., Al-Harbi, S., & Almasan, A. (2012). Mcl-1 Phosphorylation defines ABT-737 resistance that can be overcome by increased NOXA expression in leukemic B cells. *Cancer Res, 72*(12), 3069-3079. doi: 10.1158/0008-5472.CAN-11-4106

Meeusen, S., DeVay, R., Block, J., Cassidy-Stone, A., Wayson, S., McCaffery, J. M., & Nunnari, J. (2006). Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. *Cell, 127*(2), 383-395. doi: 10.1016/j.cell.2006.09.021

Meeusen, S., McCaffery, J. M., & Nunnari, J. (2004). Mitochondrial fusion intermediates revealed in vitro. *Science, 305*(5691), 1747-1752. doi: 10.1126/science.1100612

Mehrpour, M., Esclatine, A., Beau, I., & Codogno, P. (2010). Overview of macroautophagy regulation in mammalian cells. *Cell Res, 20*(7), 748-762. doi: 10.1038/cr.2010.82

Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., & Schreiber, R. D. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell, 84*(3), 431-442.

Mercer, C. A., Kaliappan, A., & Dennis, P. B. (2009). A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *Autophagy, 5*(5), 649-662.

Merino, D., Giam, M., Hughes, P. D., Siggs, O. M., Heger, K., O'Reilly, L. A., Adams, J. M., Strasser, A., Lee, E. F., Fairlie, W. D., & Bouillet, P. (2009). The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *J Cell Biol, 186*(3), 355-362. doi: 10.1083/jcb.200905153

Mestre-Escorihuela, C., Rubio-Moscardo, F., Richter, J. A., Siebert, R., Climent, J., Fresquet, V., Beltran, E., Agirre, X., Marugan, I., Marin, M., Rosenwald, A., Sugimoto, K. J., Wheat, L. M., Karran, E. L., Garcia, J. F., Sanchez, L., Prosper, F., Staudt, L. M., Pinkel, D., Dyer, M. J., & Martinez-Climent, J. A. (2007). Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. *Blood, 109*(1), 271-280. doi: 10.1182/blood-2006-06-026500

Michalak, E. M., Villunger, A., Adams, J. M., & Strasser, A. (2008). In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. *Cell Death Differ, 15*(6), 1019-1029. doi: 10.1038/cdd.2008.16

Mijaljica, D., Prescott, M., & Devenish, R. J. (2011). V-ATPase engagement in autophagic processes. *Autophagy, 7*(6), 666-668.

Ming, L., Sakaida, T., Yue, W., Jha, A., Zhang, L., & Yu, J. (2008). Sp1 and p73 activate PUMA following serum starvation. *Carcinogenesis, 29*(10), 1878-1884. doi: 10.1093/carcin/bgn150

Minn, A. J., Rudin, C. M., Boise, L. H., & Thompson, C. B. (1995). Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood, 86*(5), 1903-1910.

Mire, C. E., White, J. M., & Whitt, M. A. (2010). A spatio-temporal analysis of matrix protein and nucleocapsid trafficking during vesicular stomatitis virus uncoating. *PLoS Pathog, 6*(7), e1000994. doi: 10.1371/journal.ppat.1000994

Mire, C. E., & Whitt, M. A. (2011). The protease-sensitive loop of the vesicular stomatitis virus matrix protein is involved in virus assembly and protein translation. *Virology, 416*(1-2), 16-25. doi: 10.1016/j.virol.2011.04.013

Mitra, K., Wunder, C., Roysam, B., Lin, G., & Lippincott-Schwartz, J. (2009). A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup

and entry into S phase. *Proc Natl Acad Sci U S A, 106*(29), 11960-11965. doi: 10.1073/pnas.0904875106

Miyashita, T., & Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell, 80*(2), 293-299.

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev, 21*(22), 2861-2873. doi: 10.1101/gad.1599207

Mizushima, N., Yoshimori, T., & Levine, B. (2010). Methods in mammalian autophagy research. *Cell, 140*(3), 313-326. doi: 10.1016/j.cell.2010.01.028

Mizushima, N., Yoshimori, T., & Ohsumi, Y. (2002). Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method. *FEBS Lett, 532*(3), 450-454.

Mizushima, N., Yoshimori, T., & Ohsumi, Y. (2003). Role of the Apg12 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol, 35*(5), 553-561.

Moerdyk-Schauwecker, M., Shah, N. R., Murphy, A. M., Hastie, E., Mukherjee, P., & Grdzelishvili, V. Z. (2013). Resistance of pancreatic cancer cells to oncolytic vesicular stomatitis virus: role of type I interferon signaling. *Virology, 436*(1), 221-234. doi: 10.1016/j.virol.2012.11.014

Molica, S., Levato, D., Cascavilla, N., Levato, L., & Musto, P. (1999). Clinico-prognostic implications of simultaneous increased serum levels of soluble CD23 and beta2-microglobulin in B-cell chronic lymphocytic leukemia. *Eur J Haematol, 62*(2), 117-122.

Morales, A. A., Olsson, A., Celsing, F., Osterborg, A., Jondal, M., & Osorio, L. M. (2004). Expression and transcriptional regulation of functionally distinct Bmf isoforms in B-chronic lymphocytic leukemia cells. *Leukemia, 18*(1), 41-47. doi: 10.1038/sj.leu.2403183

Mordier, S., Deval, C., Bechet, D., Tassa, A., & Ferrara, M. (2000). Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. *J Biol Chem, 275*(38), 29900-29906. doi: 10.1074/jbc.M003633200

Mortimore, G. E., Hutson, N. J., & Surmacz, C. A. (1983). Quantitative correlation between proteolysis and macro- and microautophagy in mouse hepatocytes during starvation and refeeding. *Proc Natl Acad Sci U S A, 80*(8), 2179-2183.

Msaouel, P., Iankov, I. D., Allen, C., Morris, J. C., von Messling, V., Cattaneo, R., Koutsilieris, M., Russell, S. J., & Galanis, E. (2009). Engineered measles virus as a novel oncolytic therapy against prostate cancer. *Prostate, 69*(1), 82-91. doi: 10.1002/pros.20857

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M., & Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. *Science, 264*(5167), 1918-1921.

Munshi, C., Aarhus, R., Graeff, R., Walseth, T. F., Levitt, D., & Lee, H. C. (2000). Identification of the enzymatic active site of CD38 by site-directed mutagenesis. *J Biol Chem, 275*(28), 21566-21571. doi: 10.1074/jbc.M909365199

Nagata, M., Nakauchi, H., Nakayama, K., Nakayama, K., Loh, D., & Watanabe, T. (1996). Apoptosis during an early stage of nephrogenesis induces renal hypoplasia in bcl-2-deficient mice. *Am J Pathol, 148*(5), 1601-1611.

Nagata, S. (1997). Apoptosis by death factor. *Cell, 88*(3), 355-365.

Naniche, D., Varior-Krishnan, G., Cervoni, F., Wild, T. F., Rossi, B., Rabourdin-Combe, C., & Gerlier, D. (1993). Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J Virol, 67*(10), 6025-6032.

Naumann, U., Schmidt, F., Wick, W., Frank, B., Weit, S., Gillissen, B., Daniel, P., & Weller, M. (2003). Adenoviral natural born killer gene therapy for malignant glioma. *Hum Gene Ther, 14*(13), 1235-1246. doi: 10.1089/104303403767740777

Nguyen, M., Marcellus, R. C., Roulston, A., Watson, M., Serfass, L., Murthy Madiraju, S. R., Goulet, D., Viallet, J., Belec, L., Billot, X., Acoca, S., Purisima, E., Wiegmans, A., Cluse, L., Johnstone, R. W., Beauparlant, P., & Shore, G. C. (2007). Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A, 104*(49), 19512-19517. doi: 10.1073/pnas.0709443104

Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., & Wang, X. (2003). Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev, 17*(12), 1475-1486. doi: 10.1101/gad.1093903

Niu, X., Li, S., Wei, F., Huang, J., Wu, G., Xu, L., Xu, D., & Wang, S. (2012). Apogossypolone induces autophagy and apoptosis in breast cancer MCF-7 cells in vitro and in vivo. *Breast Cancer*. doi: 10.1007/s12282-012-0372-z

Norman, K. L., Coffey, M. C., Hirasawa, K., Demetrick, D. J., Nishikawa, S. G., DiFrancesco, L. M., Strong, J. E., & Lee, P. W. (2002). Reovirus oncolysis of human breast cancer. *Hum Gene Ther, 13*(5), 641-652. doi: 10.1089/10430340252837233

Norman, K. L., & Lee, P. W. (2000). Reovirus as a novel oncolytic agent. *J Clin Invest, 105*(8), 1035-1038. doi: 10.1172/JCI9871

Notte, A., Leclere, L., & Michiels, C. (2011). Autophagy as a mediator of chemotherapy-induced cell death in cancer. *Biochem Pharmacol, 82*(5), 427-434. doi: 10.1016/j.bcp.2011.06.015

Nyhan, M. J., O'Donovan, T. R., Elzinga, B., Crowley, L. C., O'Sullivan, G. C., & McKenna, S. L. (2012). The BH3 mimetic HA14-1 enhances 5-fluorouracil-induced autophagy and type II cell death in oesophageal cancer cells. *Br J Cancer, 106*(4), 711-718. doi: 10.1038/bjc.2011.604

O'Brien, S. M., Claxton, D. F., Crump, M., Faderl, S., Kipps, T., Keating, M. J., Viallet, J., & Cheson, B. D. (2009). Phase I study of obatoclax mesylate (GX15-070), a small molecule pan-Bcl-2 family antagonist, in patients with advanced chronic lymphocytic leukemia. *Blood, 113*(2), 299-305. doi: 10.1182/blood-2008-02-137943

O'Connor, L., Strasser, A., O'Reilly, L. A., Hausmann, G., Adams, J. M., Cory, S., & Huang, D. C. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J, 17*(2), 384-395. doi: 10.1093/emboj/17.2.384

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., & Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science, 288*(5468), 1053-1058.

Odero, M. D., Soto, J. L., Matutes, E., Martin-Subero, J. I., Zudaire, I., Rao, P. H., Cigudosa, J. C., Ardanaz, M. T., Chaganti, R. S., Perucho, M., & Calasanz, M. J. (2001). Comparative genomic hybridization and amplotyping by arbitrarily primed PCR in stage A B-CLL. *Cancer Genet Cytogenet, 130*(1), 8-13.

Ogino, T., & Banerjee, A. K. (2007). Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Mol Cell, 25*(1), 85-97. doi: 10.1016/j.molcel.2006.11.013

Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., & Rosenberg, S. H. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature, 435*(7042), 677-681. doi: 10.1038/nature03579

Opferman, J. T., & Korsmeyer, S. J. (2003). Apoptosis in the development and maintenance of the immune system. *Nat Immunol, 4*(5), 410-415. doi: 10.1038/ni0503-410

Oppenheim, R. W., Flavell, R. A., Vinsant, S., Prevette, D., Kuan, C. Y., & Rakic, P. (2001). Programmed cell death of developing mammalian neurons after genetic deletion of caspases. *J Neurosci, 21*(13), 4752-4760.

Oppermann, M., Geilen, C. C., Fecker, L. F., Gillissen, B., Daniel, P. T., & Eberle, J. (2005). Caspase-independent induction of apoptosis in human melanoma cells by the proapoptotic Bcl-2-related protein Nbk / Bik. *Oncogene, 24*(49), 7369-7380. doi: 10.1038/sj.onc.1208890

Orvedahl, A., Alexander, D., Talloczy, Z., Sun, Q., Wei, Y., Zhang, W., Burns, D., Leib, D. A., & Levine, B. (2007). HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe, 1*(1), 23-35. doi: 10.1016/j.chom.2006.12.001

Orvedahl, A., & Levine, B. (2009). Autophagy in Mammalian antiviral immunity. *Curr Top Microbiol Immunol, 335*, 267-285. doi: 10.1007/978-3-642-00302-8_13

Oscier, D. G., Gardiner, A. C., Mould, S. J., Glide, S., Davis, Z. A., Ibbotson, R. E., Corcoran, M. M., Chapman, R. M., Thomas, P. W., Copplestone, J. A., Orchard, J. A., & Hamblin, T. J. (2002). Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood, 100*(4), 1177-1184.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: a review of apoptosis, autophagy

and programmed necrosis. *Cell Prolif, 45*(6), 487-498. doi: 10.1111/j.1365-2184.2012.00845.x

Paik, P. K., Rudin, C. M., Brown, A., Rizvi, N. A., Takebe, N., Travis, W., James, L., Ginsberg, M. S., Juergens, R., Markus, S., Tyson, L., Subzwari, S., Kris, M. G., & Krug, L. M. (2010). A phase I study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in solid tumor malignancies. *Cancer Chemother Pharmacol, 66*(6), 1079-1085. doi: 10.1007/s00280-010-1265-5

Pangalis, G. A., Vassilakopoulos, T. P., Dimopoulou, M. N., Siakantaris, M. P., Kontopidou, F. N., & Angelopoulou, M. K. (2002). B-chronic lymphocytic leukemia: practical aspects. *Hematol Oncol, 20*(3), 103-146. doi: 10.1002/hon.696

Paoluzzi, L., Gonen, M., Gardner, J. R., Mastrella, J., Yang, D., Holmlund, J., Sorensen, M., Leopold, L., Manova, K., Marcucci, G., Heaney, M. L., & O'Connor, O. A. (2008). Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in in vitro and in vivo models of B-cell lymphoma. *Blood, 111*(11), 5350-5358. doi: 10.1182/blood-2007-12-129833

Parato, K. A., Senger, D., Forsyth, P. A., & Bell, J. C. (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer, 5*(12), 965-976. doi: 10.1038/nrc1750

Park, H. H., & Wu, H. (2006). Crystal structure of RAIDD death domain implicates potential mechanism of PIDDosome assembly. *J Mol Biol, 357*(2), 358-364. doi: 10.1016/j.jmb.2005.12.082

Park, W. H., Han, Y. W., Kim, S. H., & Kim, S. Z. (2007). An ROS generator, antimycin A, inhibits the growth of HeLa cells via apoptosis. *J Cell Biochem, 102*(1), 98-109. doi: 10.1002/jcb.21280

Parkinson, N., Ince, P. G., Smith, M. O., Highley, R., Skibinski, G., Andersen, P. M., Morrison, K. E., Pall, H. S., Hardiman, O., Collinge, J., Shaw, P. J., Fisher, E. M., Study, M. R. C. Proteomics in ALS, & Consortium, F. ReJA. (2006). ALS phenotypes with mutations in CHMP2B (charged multivesicular body protein 2B). *Neurology, 67*(6), 1074-1077. doi: 10.1212/01.wnl.0000231510.89311.8b

Pattingre, S., & Levine, B. (2006). Bcl-2 inhibition of autophagy: a new route to cancer? *Cancer Res, 66*(6), 2885-2888. doi: 10.1158/0008-5472.CAN-05-4412

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D., & Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell, 122*(6), 927-939. doi: 10.1016/j.cell.2005.07.002

Peng, K. W., Hadac, E. M., Anderson, B. D., Myers, R., Harvey, M., Greiner, S. M., Soeffker, D., Federspiel, M. J., & Russell, S. J. (2006). Pharmacokinetics of oncolytic measles virotherapy: eventual equilibrium between virus and tumor in an ovarian cancer xenograft model. *Cancer Gene Ther, 13*(8), 732-738. doi: 10.1038/sj.cgt.7700948

Pepper, C., Hoy, T., & Bentley, P. (1998). Elevated Bcl-2/Bax are a consistent feature of apoptosis resistance in B-cell chronic lymphocytic leukaemia and

are correlated with in vivo chemoresistance. *Leuk Lymphoma, 28*(3-4), 355-361. doi: 10.3109/10428199809092690

Perez-Galan, P., Roue, G., Villamor, N., Campo, E., & Colomer, D. (2007). The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood, 109*(10), 4441-4449. doi: 10.1182/blood-2006-07-034173

Perez, K., & Winer, E. S. (2011). Chronic lymphocytic leukemia: something old, something new and something borrowed. *Med Health R I, 94*(1), 15-18.

Pestka, S., Krause, C. D., & Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunol Rev, 202*, 8-32. doi: 10.1111/j.0105-2896.2004.00204.x

Peter, M. E., & Krammer, P. H. (2003). The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ, 10*(1), 26-35. doi: 10.1038/sj.cdd.4401186

Petersen, J. M., Her, L. S., Varvel, V., Lund, E., & Dahlberg, J. E. (2000). The matrix protein of vesicular stomatitis virus inhibits nucleocytoplasmic transport when it is in the nucleus and associated with nuclear pore complexes. *Mol Cell Biol, 20*(22), 8590-8601.

Platanias, L. C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol, 5*(5), 375-386. doi: 10.1038/nri1604

Post, D. E., & Van Meir, E. G. (2003). A novel hypoxia-inducible factor (HIF) activated oncolytic adenovirus for cancer therapy. *Oncogene, 22*(14), 2065-2072. doi: 10.1038/sj.onc.1206464

Prestwich, R. J., Errington, F., Ilett, E. J., Morgan, R. S., Scott, K. J., Kottke, T., Thompson, J., Morrison, E. E., Harrington, K. J., Pandha, H. S., Selby, P. J., Vile, R. G., & Melcher, A. A. (2008). Tumor infection by oncolytic reovirus primes adaptive antitumor immunity. *Clin Cancer Res, 14*(22), 7358-7366. doi: 10.1158/1078-0432.CCR-08-0831

Pruefer, F. G., Lizarraga, F., Maldonado, V., & Melendez-Zajgla, J. (2008). Participation of Omi Htra2 serine-protease activity in the apoptosis induced by cisplatin on SW480 colon cancer cells. *J Chemother, 20*(3), 348-354.

Puthalakath, H., Villunger, A., O'Reilly, L. A., Beaumont, J. G., Coultas, L., Cheney, R. E., Huang, D. C., & Strasser, A. (2001). Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science, 293*(5536), 1829-1832. doi: 10.1126/science.1062257

Qin, H., Srinivasula, S. M., Wu, G., Fernandes-Alnemri, T., Alnemri, E. S., & Shi, Y. (1999). Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1. *Nature, 399*(6736), 549-557. doi: 10.1038/21124

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E. L., Mizushima, N., Ohsumi, Y., Cattoretti, G., & Levine, B. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest, 112*(12), 1809-1820. doi: 10.1172/JCI20039

Quiroz, E., Moreno, N., Peralta, P. H., & Tesh, R. B. (1988). A human case of encephalitis associated with vesicular stomatitis virus (Indiana serotype) infection. *Am J Trop Med Hyg, 39*(3), 312-314.

Rai, K. R., Wasil, T., Iqbal, U., Driscoll, N., Patel, D., Janson, D., & Mehrotra, B. (2004). Clinical staging and prognostic markers in chronic lymphocytic leukemia. *Hematol Oncol Clin North Am, 18*(4), 795-805, vii. doi: 10.1016/j.hoc.2004.04.001

Raiborg, C., & Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature, 458*(7237), 445-452. doi: 10.1038/nature07961

Rajani, K. R., Pettit Kneller, E. L., McKenzie, M. O., Horita, D. A., Chou, J. W., & Lyles, D. S. (2012). Complexes of vesicular stomatitis virus matrix protein with host Rae1 and Nup98 involved in inhibition of host transcription. *PLoS Pathog, 8*(9), e1002929. doi: 10.1371/journal.ppat.1002929

Rambold, A. S., & Lippincott-Schwartz, J. (2011). Mechanisms of mitochondria and autophagy crosstalk. *Cell Cycle, 10*(23), 4032-4038. doi: 10.4161/cc.10.23.18384

Ranger, A. M., Zha, J., Harada, H., Datta, S. R., Danial, N. N., Gilmore, A. P., Kutok, J. L., Le Beau, M. M., Greenberg, M. E., & Korsmeyer, S. J. (2003). Bad-deficient mice develop diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A, 100*(16), 9324-9329. doi: 10.1073/pnas.1533446100

Ray, J. E., Garcia, J., Jurisicova, A., & Caniggia, I. (2010). Mtd/Bok takes a swing: proapoptotic Mtd/Bok regulates trophoblast cell proliferation during human placental development and in preeclampsia. *Cell Death Differ, 17*(5), 846-859. doi: 10.1038/cdd.2009.167

Redaelli, A., Laskin, B. L., Stephens, J. M., Botteman, M. F., & Pashos, C. L. (2004). The clinical and epidemiological burden of chronic lymphocytic leukaemia. *Eur J Cancer Care (Engl), 13*(3), 279-287. doi: 10.1111/j.1365-2354.2004.00489.x

Reed, J. C., Stein, C., Subasinghe, C., Haldar, S., Croce, C. M., Yum, S., & Cohen, J. (1990). Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. *Cancer Res, 50*(20), 6565-6570.

Robak, T. (2012). Rituximab for chronic lymphocytic leukemia. *Expert Opin Biol Ther, 12*(4), 503-515. doi: 10.1517/14712598.2012.665444

Roberts, A., Buonocore, L., Price, R., Forman, J., & Rose, J. K. (1999). Attenuated vesicular stomatitis viruses as vaccine vectors. *J Virol, 73*(5), 3723-3732.

Roberts, A. W., Seymour, J. F., Brown, J. R., Wierda, W. G., Kipps, T. J., Khaw, S. L., Carney, D. A., He, S. Z., Huang, D. C., Xiong, H., Cui, Y., Busman, T. A., McKeegan, E. M., Krivoshik, A. P., Enschede, S. H., & Humerickhouse, R. (2012). Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol, 30*(5), 488-496. doi: 10.1200/JCO.2011.34.7898

Rodriguez L.L., Nichol S.T., Webster R.G., Granoff A.,. (1999). *Encyclopedia of Virology* (2nd ed.). London: Academic Press.

Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., & Henderson, D. R. (1997). Prostate attenuated replication competent

adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res, 57*(13), 2559-2563.

Rosenwald, A., Chuang, E. Y., Davis, R. E., Wiestner, A., Alizadeh, A. A., Arthur, D. C., Mitchell, J. B., Marti, G. E., Fowler, D. H., Wilson, W. H., & Staudt, L. M. (2004). Fludarabine treatment of patients with chronic lymphocytic leukemia induces a p53-dependent gene expression response. *Blood, 104*(5), 1428-1434. doi: 10.1182/blood-2003-09-3236

Russell, S. J., Peng, K. W., & Bell, J. C. (2012). Oncolytic virotherapy. *Nat Biotechnol, 30*(7), 658-670. doi: 10.1038/nbt.2287

Saka, B., Aktan, M., Sami, U., Oner, D., Sanem, O., & Dincol, G. (2006). Prognostic importance of soluble CD23 in B-cell chronic lymphocytic leukemia. *Clin Lab Haematol, 28*(1), 30-35. doi: 10.1111/j.1365-2257.2006.00750.x

Salvesen, G. S. (2002). Caspases and apoptosis. *Essays Biochem, 38*, 9-19.

Salvesen, G. S., & Dixit, V. M. (1999). Caspase activation: the induced-proximity model. *Proc Natl Acad Sci U S A, 96*(20), 10964-10967.

Salvesen, G. S., & Duckett, C. S. (2002). IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol, 3*(6), 401-410. doi: 10.1038/nrm830

Santel, A., & Fuller, M. T. (2001). Control of mitochondrial morphology by a human mitofusin. *J Cell Sci, 114*(Pt 5), 867-874.

Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., & Fesik, S. W. (1997). Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science, 275*(5302), 983-986.

Sayers, T. J. (2011). Targeting the extrinsic apoptosis signaling pathway for cancer therapy. *Cancer Immunol Immunother, 60*(8), 1173-1180. doi: 10.1007/s00262-011-1008-4

Scheid, M. P., Schubert, K. M., & Duronio, V. (1999). Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. *J Biol Chem, 274*(43), 31108-31113.

Schimmer, A. D., O'Brien, S., Kantarjian, H., Brandwein, J., Cheson, B. D., Minden, M. D., Yee, K., Ravandi, F., Giles, F., Schuh, A., Gupta, V., Andreeff, M., Koller, C., Chang, H., Kamel-Reid, S., Berger, M., Viallet, J., & Borthakur, G. (2008). A phase I study of the pan bcl-2 family inhibitor obatoclax mesylate in patients with advanced hematologic malignancies. *Clin Cancer Res, 14*(24), 8295-8301. doi: 10.1158/1078-0432.CCR-08-0999

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol, 75*(2), 163-189. doi: 10.1189/jlb.0603252

Schuler, M., & Green, D. R. (2001). Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans, 29*(Pt 6), 684-688.

Seo, Y. W., Shin, J. N., Ko, K. H., Cha, J. H., Park, J. Y., Lee, B. R., Yun, C. W., Kim, Y. M., Seol, D. W., Kim, D. W., Yin, X. M., & Kim, T. H. (2003). The molecular mechanism of Noxa-induced mitochondrial dysfunction in p53-mediated cell death. *J Biol Chem, 278*(48), 48292-48299. doi: 10.1074/jbc.M308785200

Shanshal, M., & Haddad, R. Y. (2012). Chronic lymphocytic leukemia. *Dis Mon, 58*(4), 153-167. doi: 10.1016/j.disamonth.2012.01.009

Shen, H. M., & Codogno, P. (2011). Autophagic cell death: Loch Ness monster or endangered species? *Autophagy, 7*(5), 457-465.

Shen, S., Kepp, O., & Kroemer, G. (2012). The end of autophagic cell death? *Autophagy, 8*(1), 1-3. doi: 10.4161/auto.8.1.16618

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell, 9*(3), 459-470.

Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B., & Tsujimoto, Y. (2004). Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol, 6*(12), 1221-1228. doi: 10.1038/ncb1192

Shore, G. C., & Viallet, J. (2005). Modulating the bcl-2 family of apoptosis suppressors for potential therapeutic benefit in cancer. *Hematology Am Soc Hematol Educ Program*, 226-230. doi: 10.1182/asheducation-2005.1.226

Siegel, R., Ward, E., Brawley, O., & Jemal, A. (2011). Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin, 61*(4), 212-236. doi: 10.3322/caac.20121

Sieghart, W., Losert, D., Strommer, S., Cejka, D., Schmid, K., Rasoul-Rockenschaub, S., Bodingbauer, M., Crevenna, R., Monia, B. P., Peck-Radosavljevic, M., & Wacheck, V. (2006). Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. *J Hepatol, 44*(1), 151-157. doi: 10.1016/j.jhep.2005.09.010

Sinha, S., & Levine, B. (2008). The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene, 27 Suppl 1*, S137-148. doi: 10.1038/onc.2009.51

Skibinski, G., Parkinson, N. J., Brown, J. M., Chakrabarti, L., Lloyd, S. L., Hummerich, H., Nielsen, J. E., Hodges, J. R., Spillantini, M. G., Thusgaard, T., Brandner, S., Brun, A., Rossor, M. N., Gade, A., Johannsen, P., Sorensen, S. A., Gydesen, S., Fisher, E. M., & Collinge, J. (2005). Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. *Nat Genet, 37*(8), 806-808. doi: 10.1038/ng1609

Smolewski, P., & Robak, T. (2011). Inhibitors of apoptosis proteins (IAPs) as potential molecular targets for therapy of hematological malignancies. *Curr Mol Med, 11*(8), 633-649.

Smoot, R. L., Blechacz, B. R., Werneburg, N. W., Bronk, S. F., Sinicrope, F. A., Sirica, A. E., & Gores, G. J. (2010). A Bax-mediated mechanism for obatoclax-induced apoptosis of cholangiocarcinoma cells. *Cancer Res, 70*(5), 1960-1969. doi: 10.1158/0008-5472.CAN-09-3535

Stilgenbauer, S., Liebisch, P., James, M. R., Schroder, M., Schlegelberger, B., Fischer, K., Bentz, M., Lichter, P., & Dohner, H. (1996). Molecular cytogenetic delineation of a novel critical genomic region in chromosome bands 11q22.3-923.1 in lymphoproliferative disorders. *Proc Natl Acad Sci U S A, 93*(21), 11837-11841.

Stoeckel, J., & Hay, J. G. (2006). Drug evaluation: Reolysin--wild-type reovirus as a cancer therapeutic. *Curr Opin Mol Ther, 8*(3), 249-260.

Stojdl, D. F., Lichty, B. D., tenOever, B. R., Paterson, J. M., Power, A. T., Knowles, S., Marius, R., Reynard, J., Poliquin, L., Atkins, H., Brown, E. G., Durbin, R. K., Durbin, J. E., Hiscott, J., & Bell, J. C. (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell, 4*(4), 263-275.

Strasser, A. (2005). The role of BH3-only proteins in the immune system. *Nat Rev Immunol, 5*(3), 189-200. doi: 10.1038/nri1568

Suen, D. F., Norris, K. L., & Youle, R. J. (2008). Mitochondrial dynamics and apoptosis. *Genes Dev, 22*(12), 1577-1590. doi: 10.1101/gad.1658508

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., & Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature, 397*(6718), 441-446. doi: 10.1038/17135

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., & Ohsumi, Y. (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J, 20*(21), 5971-5981. doi: 10.1093/emboj/20.21.5971

Tahir, S. K., Wass, J., Joseph, M. K., Devanarayan, V., Hessler, P., Zhang, H., Elmore, S. W., Kroeger, P. E., Tse, C., Rosenberg, S. H., & Anderson, M. G. (2010). Identification of expression signatures predictive of sensitivity to the Bcl-2 family member inhibitor ABT-263 in small cell lung carcinoma and leukemia/lymphoma cell lines. *Mol Cancer Ther, 9*(3), 545-557. doi: 10.1158/1535-7163.MCT-09-0651

Takahashi, Y., Meyerkord, C. L., Hori, T., Runkle, K., Fox, T. E., Kester, M., Loughran, T. P., & Wang, H. G. (2011). Bif-1 regulates Atg9 trafficking by mediating the fission of Golgi membranes during autophagy. *Autophagy, 7*(1), 61-73.

Takamura, A., Komatsu, M., Hara, T., Sakamoto, A., Kishi, C., Waguri, S., Eishi, Y., Hino, O., Tanaka, K., & Mizushima, N. (2011). Autophagy-deficient mice develop multiple liver tumors. *Genes Dev, 25*(8), 795-800. doi: 10.1101/gad.2016211

Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., Negishi, H., Kikuchi, H., Sasaki, S., Imai, K., Shibue, T., Honda, K., & Taniguchi, T. (2003). Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature, 424*(6948), 516-523. doi: 10.1038/nature01850

Talloczy, Z., Jiang, W., Virgin, H. W. th, Leib, D. A., Scheuner, D., Kaufman, R. J., Eskelinen, E. L., & Levine, B. (2002). Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. *Proc Natl Acad Sci U S A, 99*(1), 190-195. doi: 10.1073/pnas.012485299

Tanida, I., Tanida-Miyake, E., Komatsu, M., Ueno, T., & Kominami, E. (2002). Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. *J Biol Chem, 277*(16), 13739-13744. doi: 10.1074/jbc.M200385200

Tatsuo, H., Ono, N., Tanaka, K., & Yanagi, Y. (2000). SLAM (CDw150) is a cellular receptor for measles virus. *Nature, 406*(6798), 893-897. doi: 10.1038/35022579

Terstegen, L., Gatsios, P., Ludwig, S., Pleschka, S., Jahnen-Dechent, W., Heinrich, P. C., & Graeve, L. (2001). The vesicular stomatitis virus matrix protein inhibits glycoprotein 130-dependent STAT activation. *J Immunol, 167*(9), 5209-5216.

Tilly, J. L. (2001). Commuting the death sentence: how oocytes strive to survive. *Nat Rev Mol Cell Biol, 2*(11), 838-848. doi: 10.1038/35099086

Tinel, A., & Tschopp, J. (2004). The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science, 304*(5672), 843-846. doi: 10.1126/science.1095432

Tischner, D., Woess, C., Ottina, E., & Villunger, A. (2010). Bcl-2-regulated cell death signalling in the prevention of autoimmunity. *Cell Death Dis, 1*, e48. doi: 10.1038/cddis.2010.27

Treanor, B. (2012). B-cell receptor: from resting state to activate. *Immunology, 136*(1), 21-27. doi: 10.1111/j.1365-2567.2012.03564.x

Trudel, S., Li, Z. H., Rauw, J., Tiedemann, R. E., Wen, X. Y., & Stewart, A. K. (2007). Preclinical studies of the pan-Bcl inhibitor obatoclax (GX015-070) in multiple myeloma. *Blood, 109*(12), 5430-5438. doi: 10.1182/blood-2006-10-047951

Tse, C., Shoemaker, A. R., Adickes, J., Anderson, M. G., Chen, J., Jin, S., Johnson, E. F., Marsh, K. C., Mitten, M. J., Nimmer, P., Roberts, L., Tahir, S. K., Xiao, Y., Yang, X., Zhang, H., Fesik, S., Rosenberg, S. H., & Elmore, S. W. (2008). ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res, 68*(9), 3421-3428. doi: 10.1158/0008-5472.CAN-07-5836

Tsuchiya, K. D. (2011). Fluorescence in situ hybridization. *Clin Lab Med, 31*(4), 525-542, vii-viii. doi: 10.1016/j.cll.2011.08.011

Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., & Croce, C. M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science, 226*(4678), 1097-1099.

Tsujimoto, Y., & Shimizu, S. (2005). Another way to die: autophagic programmed cell death. *Cell Death Differ, 12 Suppl 2*, 1528-1534. doi: 10.1038/sj.cdd.4401777

Tzung, S. P., Kim, K. M., Basanez, G., Giedt, C. D., Simon, J., Zimmerberg, J., Zhang, K. Y., & Hockenbery, D. M. (2001). Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. *Nat Cell Biol, 3*(2), 183-191. doi: 10.1038/35055095

Umetani, N., Fujimoto, A., Takeuchi, H., Shinozaki, M., Bilchik, A. J., & Hoon, D. S. (2004). Allelic imbalance of APAF-1 locus at 12q23 is related to progression of colorectal carcinoma. *Oncogene, 23*(50), 8292-8300. doi: 10.1038/sj.onc.1208022

van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P. E., Willis, S. N., Scott, C. L., Day, C. L., Cory, S., Adams, J. M., Roberts, A. W., & Huang, D. C. (2006). The BH3 mimetic ABT-737 targets selective

Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell, 10*(5), 389-399. doi: 10.1016/j.ccr.2006.08.027

van der Veer, M. S., de Weers, M., van Kessel, B., Bakker, J. M., Wittebol, S., Parren, P. W., Lokhorst, H. M., & Mutis, T. (2011). The therapeutic human CD38 antibody daratumumab improves the anti-myeloma effect of newly emerging multi-drug therapies. *Blood Cancer J, 1*(10), e41. doi: 10.1038/bcj.2011.42

van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R., & Vandenabeele, P. (2001). Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ, 8*(12), 1136-1142. doi: 10.1038/sj.cdd.4400944

Veis, D. J., Sorenson, C. M., Shutter, J. R., & Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell, 75*(2), 229-240.

Verhagen, A. M., Silke, J., Ekert, P. G., Pakusch, M., Kaufmann, H., Connolly, L. M., Day, C. L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R. L., Simpson, R. J., & Vaux, D. L. (2002). HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem, 277*(1), 445-454. doi: 10.1074/jbc.M109891200

Villunger, A., Michalak, E. M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M. J., Adams, J. M., & Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science, 302*(5647), 1036-1038. doi: 10.1126/science.1090072

von Kobbe, C., van Deursen, J. M., Rodrigues, J. P., Sitterlin, D., Bachi, A., Wu, X., Wilm, M., Carmo-Fonseca, M., & Izaurralde, E. (2000). Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Mol Cell, 6*(5), 1243-1252.

Voss, V., Senft, C., Lang, V., Ronellenfitsch, M. W., Steinbach, J. P., Seifert, V., & Kogel, D. (2010). The pan-Bcl-2 inhibitor (-)-gossypol triggers autophagic cell death in malignant glioma. *Mol Cancer Res, 8*(7), 1002-1016. doi: 10.1158/1541-7786.MCR-09-0562

Vucic, D., & Fairbrother, W. J. (2007). The inhibitor of apoptosis proteins as therapeutic targets in cancer. *Clin Cancer Res, 13*(20), 5995-6000. doi: 10.1158/1078-0432.CCR-07-0729

Wagner RR, Rose JK. (1996). *Fields Virology* (K. D. BN, Howley PM Ed. 3rd Edition ed.). Philadelphia: Lipincott–Raven Publishers.

Wang, B. X., & Fish, E. N. (2012). The yin and yang of viruses and interferons. *Trends Immunol, 33*(4), 190-197. doi: 10.1016/j.it.2012.01.004

Wang, G., Nikolovska-Coleska, Z., Yang, C. Y., Wang, R., Tang, G., Guo, J., Shangary, S., Qiu, S., Gao, W., Yang, D., Meagher, J., Stuckey, J., Krajewski, K., Jiang, S., Roller, P. P., Abaan, H. O., Tomita, Y., & Wang, S. (2006). Structure-based design of potent small-molecule inhibitors of anti-apoptotic Bcl-2 proteins. *J Med Chem, 49*(21), 6139-6142. doi: 10.1021/jm060460o

Wang, H., Kadlecek, T. A., Au-Yeung, B. B., Goodfellow, H. E., Hsu, L. Y., Freedman, T. S., & Weiss, A. (2010). ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harb Perspect Biol, 2*(5), a002279. doi: 10.1101/cshperspect.a002279

Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., & Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science, 292*(5517), 727-730. doi: 10.1126/science.1059108

Wei, Y., Pattingre, S., Sinha, S., Bassik, M., & Levine, B. (2008). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol Cell, 30*(6), 678-688. doi: 10.1016/j.molcel.2008.06.001

Weng, C., Li, Y., Xu, D., Shi, Y., & Tang, H. (2005). Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J Biol Chem, 280*(11), 10491-10500. doi: 10.1074/jbc.M412819200

Whelan, S. P. (2008). *Encyclopedia of Virology* (3rd edition ed.): Academic Press.

White, E. (2012). Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer, 12*(6), 401-410. doi: 10.1038/nrc3262

Wilkinson, J. C., Wilkinson, A. S., Scott, F. L., Csomos, R. A., Salvesen, G. S., & Duckett, C. S. (2004). Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. *J Biol Chem, 279*(49), 51082-51090. doi: 10.1074/jbc.M408655200

Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., & Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev, 19*(11), 1294-1305. doi: 10.1101/gad.1304105

Willis, S. N., Fletcher, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czabotar, P. E., Ierino, H., Lee, E. F., Fairlie, W. D., Bouillet, P., Strasser, A., Kluck, R. M., Adams, J. M., & Huang, D. C. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science, 315*(5813), 856-859. doi: 10.1126/science.1133289

Wilson-Annan, J., O'Reilly, L. A., Crawford, S. A., Hausmann, G., Beaumont, J. G., Parma, L. P., Chen, L., Lackmann, M., Lithgow, T., Hinds, M. G., Day, C. L., Adams, J. M., & Huang, D. C. (2003). Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. *J Cell Biol, 162*(5), 877-887. doi: 10.1083/jcb.200302144

Wilson, N. S., Dixit, V., & Ashkenazi, A. (2009). Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol, 10*(4), 348-355. doi: 10.1038/ni.1714

Wirawan, E., Lippens, S., Vanden Berghe, T., Romagnoli, A., Fimia, G. M., Piacentini, M., & Vandenabeele, P. (2012). Beclin1: a role in membrane dynamics and beyond. *Autophagy, 8*(1), 6-17. doi: 10.4161/auto.8.1.16645

Wong, J., Zhang, J., Si, X., Gao, G., Mao, I., McManus, B. M., & Luo, H. (2008). Autophagosome supports coxsackievirus B3 replication in host cells. *J Virol, 82*(18), 9143-9153. doi: 10.1128/JVI.00641-08

Xue, D., Shaham, S., & Horvitz, H. R. (1996). The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes Dev, 10*(9), 1073-1083.

Yamaguchi, M., Noda, N. N., Nakatogawa, H., Kumeta, H., Ohsumi, Y., & Inagaki, F. (2010). Autophagy-related protein 8 (Atg8) family interacting motif in Atg3 mediates the Atg3-Atg8 interaction and is crucial for the cytoplasm-to-vacuole targeting pathway. *J Biol Chem, 285*(38), 29599-29607. doi: 10.1074/jbc.M110.113670

Yanagi, Y. (2001). [The cellular receptor for measles virus]. *Uirusu, 51*(2), 201-208.

Yang, J. Y., Chang, C. J., Xia, W., Wang, Y., Wong, K. K., Engelman, J. A., Du, Y., Andreeff, M., Hortobagyi, G. N., & Hung, M. C. (2010). Activation of FOXO3a is sufficient to reverse mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor chemoresistance in human cancer. *Cancer Res, 70*(11), 4709-4718. doi: 10.1158/0008-5472.CAN-09-4524

Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., Bause, A., Li, Y., Stommel, J. M., Dell'antonio, G., Mautner, J., Tonon, G., Haigis, M., Shirihai, O. S., Doglioni, C., Bardeesy, N., & Kimmelman, A. C. (2011). Pancreatic cancers require autophagy for tumor growth. *Genes Dev, 25*(7), 717-729. doi: 10.1101/gad.2016111

Yang, T., Kozopas, K. M., & Craig, R. W. (1995). The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol, 128*(6), 1173-1184.

Yap, T. A., Brunetto, A., Pandha, H., Harrington, K., & Debono, J. S. (2008). Reovirus therapy in cancer: has the orphan virus found a home? *Expert Opin Investig Drugs, 17*(12), 1925-1935. doi: 10.1517/13543780802533401

Yethon, J. A., Epand, R. F., Leber, B., Epand, R. M., & Andrews, D. W. (2003). Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis. *J Biol Chem, 278*(49), 48935-48941. doi: 10.1074/jbc.M306289200

Yoon, J. H., Her, S., Kim, M., Jang, I. S., & Park, J. (2012). The expression of damage-regulated autophagy modulator 2 (DRAM2) contributes to autophagy induction. *Mol Biol Rep, 39*(2), 1087-1093. doi: 10.1007/s11033-011-0835-x

Yoshimori, T., & Noda, T. (2008). Toward unraveling membrane biogenesis in mammalian autophagy. *Curr Opin Cell Biol, 20*(4), 401-407. doi: 10.1016/j.ceb.2008.03.010

You, H., Pellegrini, M., Tsuchihara, K., Yamamoto, K., Hacker, G., Erlacher, M., Villunger, A., & Mak, T. W. (2006). FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med, 203*(7), 1657-1663. doi: 10.1084/jem.20060353

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol, 9*(1), 47-59. doi: 10.1038/nrm2308

Yu, J., Wang, Z., Kinzler, K. W., Vogelstein, B., & Zhang, L. (2003). PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci U S A, 100*(4), 1931-1936. doi: 10.1073/pnas.2627984100

Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E. H., & Lenardo, M. J. (2004). Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science, 304*(5676), 1500-1502. doi: 10.1126/science.1096645

Yue, Z., Jin, S., Yang, C., Levine, A. J., & Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A, 100*(25), 15077-15082. doi: 10.1073/pnas.2436255100

Zenz, T., Krober, A., Scherer, K., Habe, S., Buhler, A., Benner, A., Denzel, T., Winkler, D., Edelmann, J., Schwanen, C., Dohner, H., & Stilgenbauer, S. (2008). Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood, 112*(8), 3322-3329. doi: 10.1182/blood-2008-04-154070

Zha, J., Harada, H., Yang, E., Jockel, J., & Korsmeyer, S. J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell, 87*(4), 619-628.

Zhai, D., Jin, C., Satterthwait, A. C., & Reed, J. C. (2006). Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. *Cell Death Differ, 13*(8), 1419-1421. doi: 10.1038/sj.cdd.4401937

Zhong, Q., Gao, W., Du, F., & Wang, X. (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell, 121*(7), 1085-1095. doi: 10.1016/j.cell.2005.06.009

Zhong, Y., Wang, Q. J., Li, X., Yan, Y., Backer, J. M., Chait, B. T., Heintz, N., & Yue, Z. (2009). Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat Cell Biol, 11*(4), 468-476. doi: 10.1038/ncb1854

Zimmer, B., Summermatter, K., & Zimmer, G. (2013). Stability and inactivation of vesicular stomatitis virus, a prototype rhabdovirus. *Vet Microbiol, 162*(1), 78-84. doi: 10.1016/j.vetmic.2012.08.023

Zou, H., Li, Y., Liu, X., & Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem, 274*(17), 11549-11556.

CHAPTER 2

Results

Manuscript I

VSV Oncolysis in Combination With the BCL-2 Inhibitor Obatoclax Overcomes Apoptosis Resistance in Chronic Lymphocytic Leukemia

Mol Ther. 2010 Dec;18(12):2094-103

Sara Samuel[1],[2], Vanessa F Tumilasci[1],[2], Stephanie Oliere[1],[2], Thi Liên-Anh Nguyên[1], April Shamy[3], John Bell4 and John Hiscott[1],[2],[3]*

[1]*Molecular Oncology Group, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada*
[2]*Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada*
[3]*Department of Medicine, Jewish General Hospital, McGill University, Montreal, Quebec, Canada*
[4]*Ottawa Health Research Institute, University of Ottawa, Ottawa, Ontario, Canada*

SS and VFT contributed equally to this work.

* Corresponding Author

Running Title: Obatoclax Sensitizes CLL Cells to VSV Oncolysis
Keywords:

Rationale for Manuscript I

Although a number of cancer models are sensitive to the oncolytic activity of VSV-AV1, several remain partially or completely resistant to oncolytic virotherapy. Intrinsic defects, such as the aberrant expression of apoptotic proteins, significantly contribute to blocking the therapeutic effects of OVs such as VSV-AV1. CLL cells are susceptible to VSV-AV1 infection and replication but fail to respond to the lytic portion of virus life cycle. To address this issue, the combination approach of Bcl-2 inhibitor, Obatoclax, and VSV-AV1 was developed as a novel strategy for CLL therapy. We hypothesized that the Bcl-2 antagonist, a BH3-only protein mimetic, would sensitize primary CLL cells to viral oncolysis by VSV-AV1. The aims of this study were to:

1) Examine the capacity of Bcl-2 antagonist Obatolclax—currently in phase II clinical trials—to overcome CLL resistance to VSV-AV1 oncolysis

2) Elucidate the molecular mechanism of apoptotic cell death induced by the combination treatment

Abstract

In chronic lymphocytic leukemia (CLL), overexpression of antiapoptotic B-cell leukemia/lymphoma 2 (BCL-2) family members contributes to leukemogenesis by interfering with apoptosis; BCL-2 expression also impairs vesicular stomatitis virus (VSV)-mediated oncolysis of primary CLL cells. In the effort to reverse resistance to VSV-mediated oncolysis, we combined VSV with obatoclax (GX15-070)—a small-molecule BCL-2 inhibitor currently in phase 2 clinical trials—and examined the molecular mechanisms governing the in vitro and in vivo anti-tumor efficiency of combining the two agents. In combination with VSV, obatoclax synergistically induced cell death in primary CLL samples and reduced tumor growth in severe combined immunodeficient- (SCID) mice-bearing A20 lymphoma tumors. Mechanistically, the combination stimulated the mitochondrial apoptotic pathway, as reflected by caspase-3 and -9 cleavage, cytochrome c release and BAX translocation. Combination treatment triggered the release of BAX from BCL-2 and myeloid cell leukemia-1 (MCL-1) from BAK, whereas VSV infection induced NOXA expression and increased the formation of a novel BAX-NOXA heterodimer. Finally, NOXA was identified as an important inducer of VSV-obatoclax driven apoptosis via knockdown and overexpression of NOXA. These studies offer insight into the synergy between small-molecule BCL-2 inhibitors such as obatoclax and VSV as a combination strategy to overcome apoptosis resistance in CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is caused by a defect in apoptosis rather than increased proliferation of CD5+ B lymphocytes.[1,2] Resistance to cytotoxic treatments in CLL is largely due to the overexpression of antiapoptotic B-cell lymphoma-2 (BCL-2) family members BCL-2 and myeloid cell leukemia (MCL-1).[3,4] High levels of BCL-2 in CLL patients correlates to decreased overall survival and chemoresistance, whereas MCL-1 overexpression is associated with failure to achieve complete remission.[5,6,7] BCL-2 proteins are subdivided into anti- and proapoptotic classes. Prosurvival members such as BCL-2, BCL-xL, A1, and MCL-1 block apoptosis by binding to and preventing proapoptotic members such as BAX and BAK from oligomerizing and forming pores at the mitochondrial membrane that trigger mitochondrial depolarization.[8,9,10] BH-3-only proteins (BIM, tBID, PUMA, NOXA, BAD) bind to antiapoptotic members of the BCL-2 family (MCL-1, BCL-2, BCL-xL, BCL-w), resulting in the release of proapoptotic BAX and/or BAK9,11 or directly bind and activate BAX/BAK.[8]

Knowledge that overexpression of BCL-2 proteins leads to resistance in many cancers has sparked considerable interest in the development of small-molecule BCL-2 inhibitors.[12,13] Encouraging results with BCL-2 inhibitors—either alone or in combination with standard chemotherapies—have been demonstrated with various cancers, including CLL.[13,14,15] obatoclax (GX15-070)—one of the promising pan-BCL-2 inhibitors currently in clinical trials—is an indole-derived broad-spectrum inhibitor with multiple targets among the BCL-2 proteins. Obatoclax binds to the hydrophobic pocket within the BH-3-binding groove of antiapoptotic proteins such as BCL-2, MCL-1, and BCL-xL, and interferes with the ability of these proteins to interact with and negatively regulate proapoptotic BCL-2 proteins such as BAX and BAK.[16,17] In preclinical studies, obatoclax has shown cytotoxic efficacy against a variety of cancers including myeloma, breast cancer, mantle cell lymphoma, and nonsmall cell lung cancer cells (NSCLC) [16,18,19,20]

Oncolytic viruses have emerged as a potential treatment for solid tumors and hematological malignancies.[21,22,23] By exploiting tumor-specific defects in the interferon signaling pathway, vesicular stomatitis virus (VSV)—a prototypical oncolytic virus—infects and replicates specifically within cancerous cells, resulting in apoptotic cell death. Initiation of apoptosis by VSV can occur through the intrinsic mitochondrial pathway, via induction of the BH-3-only, proapoptotic protein NOXA,[25,26,27] or through the extrinsic pathway via caspase-8 and BID cleavage.[28,29]

We previously showed that the resistance of CLL cells to VSV-induced oncolysis can be overcome using a combination of VSV with small-molecule BCL-2 inhibitors.23 In the present study, we used the pan-BCL-2 family inhibitor obatoclax and characterized the mechanism governing its synergistic effect with VSV. Combination therapy triggered intrinsic apoptosis leading to caspase-9 and -3 activation, BAX translocation and cytochrome c release. The efficacy of the VSV-obatoclax combination was further demonstrated in vivo where reduced tumor progression in an A20 murine B-lymphoma xenograft model was observed. The proapoptotic protein NOXA was identified as a central inducer of apoptosis that increased the ratio of proapoptotic BAX and BAK containing complexes at the mitochondrial membrane.

Results

VSV-obatoclax combination synergistically induces cell death in primary CLL cells. To determine the lowest efficient dose of obatoclax that could be used in combination with VSV, a dose-dependent killing curve was performed in primary CD5+ CD19+ CLL cells (Figure 1a). Obatoclax had an IC50 of 640 nmol/l, lower than the IC50 (1 μmol/l) in peripheral blood mononuclear cells (PBMCs) from healthy volunteers; however at 640 nmol/l, obatoclax killed a significant amount (30%) of healthy PBMCs (Figure 1a). A lower dose of 100 nmol/l of obatoclax was sufficient to synergistically trigger cell death in 72% ($P < 0.001$) of primary CD5+ CD19+ CLL samples infected with VSV (10 multiplicity of infection), but did not induce >8% cell death in healthy PBMCs. Each treatment alone showed minimal killing activity in primary CLL cells (5 and 25% for VSV and obatoclax, respectively (Figure 1b)). The enhanced cytotoxic effect of VSV-obatoclax was not prominent with obatoclax doses <100 nmol/l. These results demonstrate synergistic cytotoxicity of CD5+ CD19+ CLL cells using the VSV-obatoclax, combination, with minimal cytotoxic effect on healthy PBMCs at 100 nmol/l obatoclax.

BCL-2 inhibits apoptosis by binding BAX, thus preventing mitochondrial pore formation and membrane permeabilization.[9,10,11] To determine the effect of obatoclax on BCL-2–BAX interaction, anti-BAX coimmunoprecipitations were performed in Karpas-422 cells, treated with increasing doses of inhibitor (0—1,000 nmol/l). Obatoclax inhibited the interaction between BCL-2 and BAX at high concentrations (>500 nmol/l), whereas at 100 nmol/l no disruption of BCL-2/BAX was observed (Figure 1c), thus demonstrating that 100 nmol/l obatoclax was suboptimal as a single treatment.

Figure 1: VSV-obatoclax combination therapy enhances cytotoxicity in CD5+ CD19+ CLL cells.

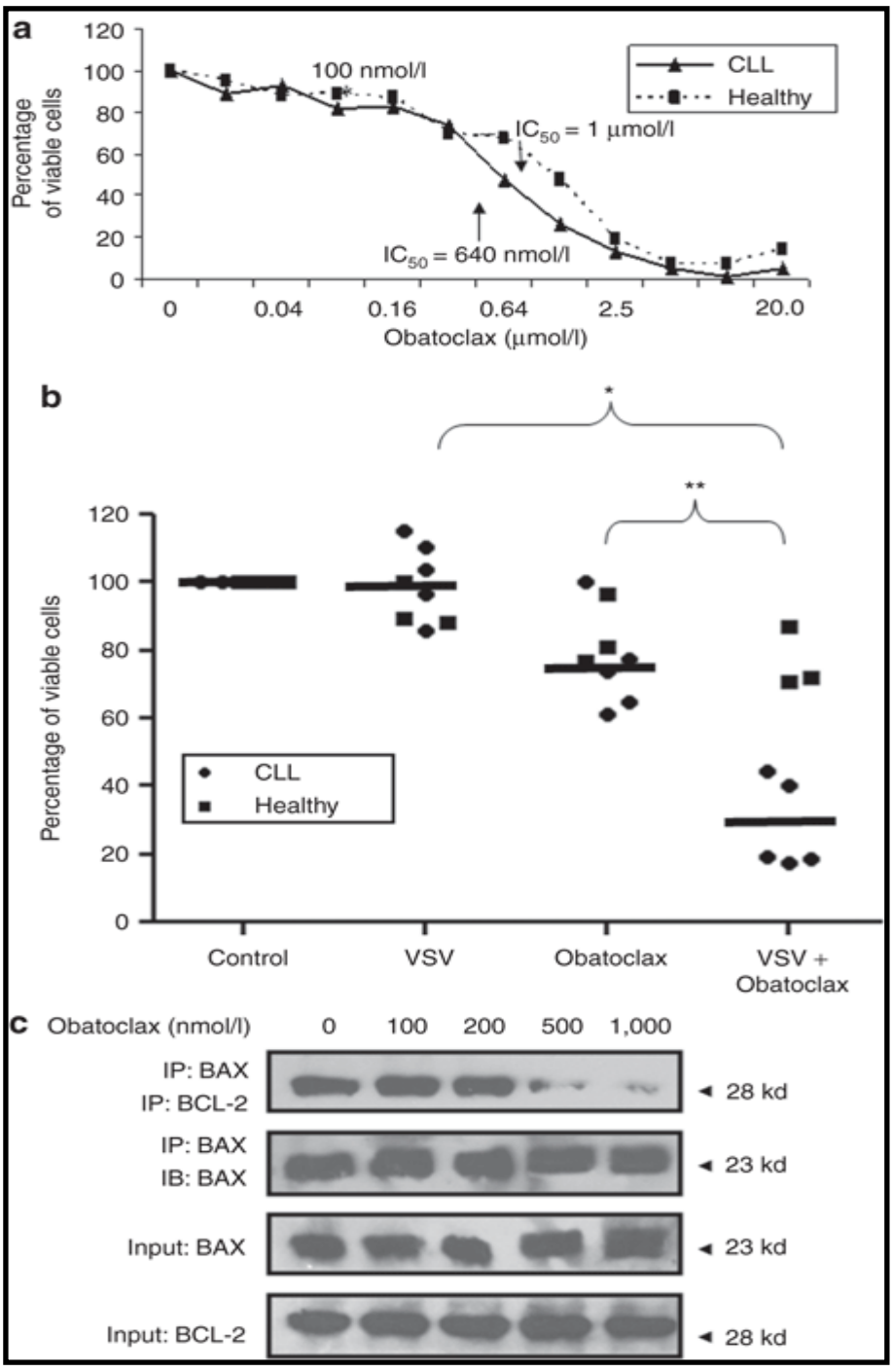

Figure 1 Legend: VSV-obatoclax combination therapy enhances cytotoxicity in CD5+ CD19+ CLL cells. (a) The IC50 of obatoclax was determined in PBMCs from CLL patients and healthy volunteers; PBMCs were treated with varying concentrations of obatoclax (0–20 μmol/l) and cell viability was assessed by MTT assay. The results are reported as percentage of viable cells; values represent the mean of quadruplicate experiments ± SD. (b) Cell viability of PBMCs from five CLL patients and four healthy volunteers was assessed by MTT assay. The results are reported on a scatter graph as percentage of viable cells (*$P < 0.01$; **$P < 0.0001$); the mean value for all patients within a group is indicated by the line. (c) Karpas-422 cells were treated with different concentrations of obatoclax (100–1,000 nmol/l) for 24 hours. Cells were lysed in 1% CHAPS lysis buffer and BAX was immunoprecipitated (IP) followed by immunoblotting (IB) with anti-BCL-2 antibody. BCL, B-cell lymphoma-2; CLL, chronic lymphocytic leukemia; PBMC, peripheral blood mononuclear cell; VSV, vesicular stomatitis virus.

Obatoclax increases VSV-induced oncolysis in A20 B-lymphoma xenograft tumors in SCID mice. After establishing the cytotoxicity of combination therapy on CLL cells in vitro, the anti-tumor effects of VSV-obatoclax were examined in vivo in Fox Chase severe combined immunodeficient (SCID) mice-bearing A20 xenograft tumors. Like Karpas-422 and primary CLL cells, A20 B-lymphoma cells overexpress BCL-2 (Figure 2a) and are partially resistant to VSV-induced apoptosis. In vitro, obatoclax treatment of A20 cells decreased viability by 10%, whereas VSV infection resulted in a 40% decrease; the combination reduced viability by 70% (Figure 2b). To determine the effect of the combination in vivo, SCID mice were injected with 1×10^6 A20 cells; when tumors were palpable at day 12, animals received obatoclax 3 mg/kg/day (intraperitoneal injection) for five consecutive days (days 12–16) and two intratumoral injections of 1×10^8 plaque-forming units of VSV at days 13 and 16 (Figure 2c). As shown in Figure 2c, tumors grew to a diameter of ~2,200 mm^3 by day 26 without treatment. Treatment with obatoclax led to a 40% decrease in tumor size compared to untreated animals. Mice receiving VSV alone exhibited 65% suppression of tumor growth compared to control animals. Tumor growth was decreased further with the VSV-obatoclax combination (80%).

Figure 2: Obatoclax treatment augments VSV-mediated oncolysis in A20 B-lymphoma xenografts in SCID mice

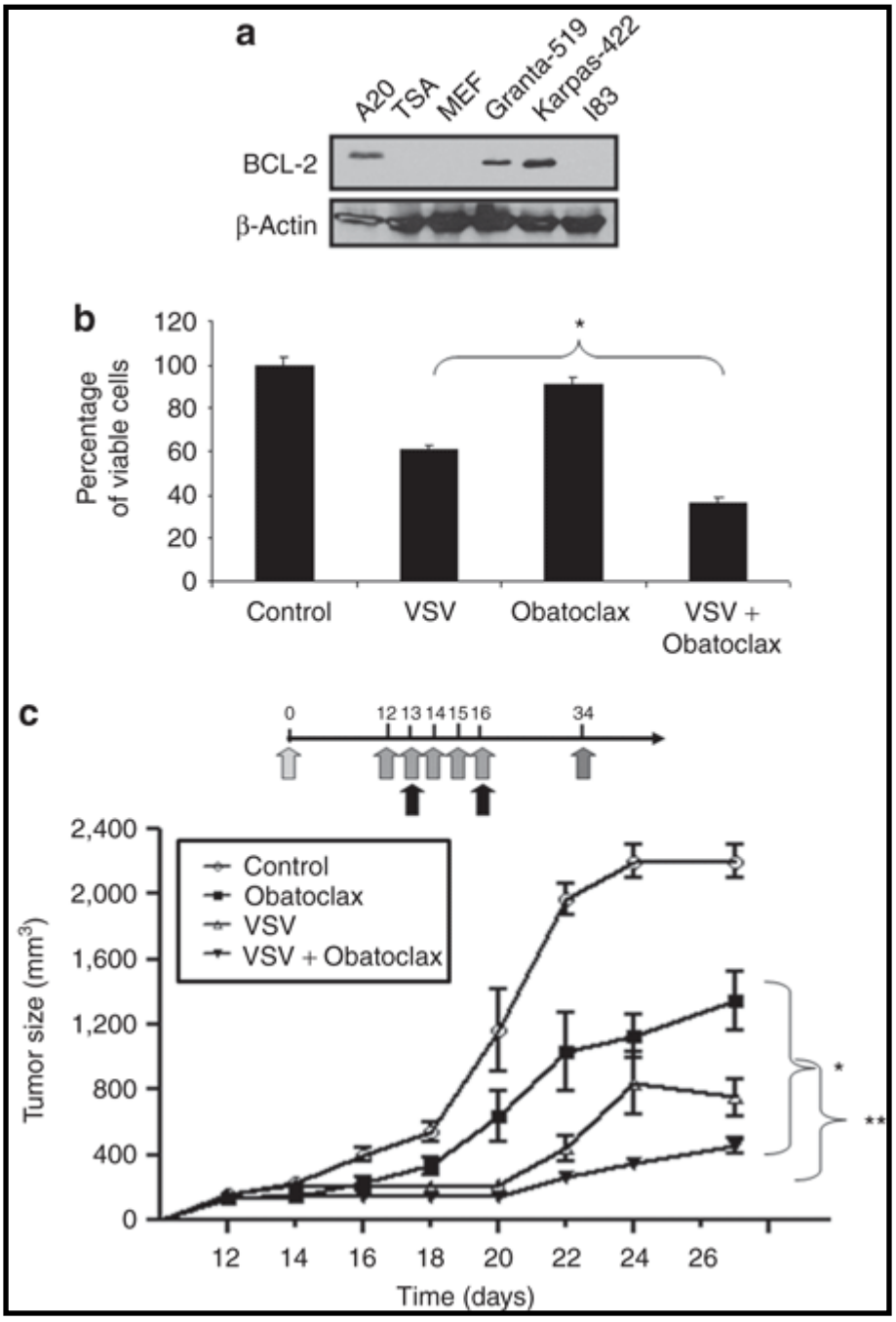

Figure 2 Legend: (a) BCL-2 expression. Proteins from six different B-lymphoma cell lines were isolated using 1% CHAPS buffer. BCL-2 expression in A20 cells compared to Grantas-519 and Karpas-422 cells was analyzed by western blot with anti-BCL-2 antibody. (b) Viability of A20. Cells were treated with or without VSV and obatoclax. At 72 hours postinfection, cell viability was assessed by MTT assay. Results are reported as percentage of viable cells ± SD; each experiment was performed in quadruplicate (*$P < 0.01$). (c) A20 murine B-lymphoma cells were inoculated into the flank of SCID mice on day 0. Mice-bearing A20 xenograft tumors received five intraperitoneal injections of obatoclax and two intratumoral VSV injections, beginning on day 12, through day 16. The stars indicate $P < 0.001$ comparing tumor size between the single and combination treatment groups. Tumor volumes were calculated as ½(length × width2) and values are expressed as the mean ± SD of tumor volume (n = 8). BCL, B-cell lymphoma-2; CLL, chronic lymphocytic leukemia; PBMC, peripheral blood mononuclear cell; SCID, severe combined immunodeficient; VSV, vesicular stomatitis virus.

VSV-obatoclax activates apoptosis through the intrinsic pathway. After establishing the efficacy of the combination therapy in vitro and in vivo, the mechanism(s) by which the individual and combination treatment induced oncolysis was evaluated. Key proteins involved in triggering the extrinsic (caspase 8) and intrinsic (caspase-9) apoptotic pathways were examined, as well as downstream effectors (BID and caspase-3). The amount of cleaved caspase-3 was increased threefold (Figure 3a, lane 4) in CLL cells with the combination compared to obatoclax alone and was increased more than fivefold (Figure 3b, lane 4) in Karpas-422 cells compared to VSV or obatoclax, whereas caspase-8 and BID cleavage were not detected following single or combination treatments (Figure 3a,b). Because of the key role for caspase-8 as an initiator of the extrinsic pathway[30] and as an activator of BID cleavage,[31] we concluded that activation of the extrinsic pathway was not involved in VSV-obatoclax induced apoptosis. In contrast, VSV-obatoclax treatment effectively induced caspase-9 cleavage (Figure 3a,b, lane 4), the initiator for the intrinsic mitochondrial pathway.[32]

Figure 3: VSV-obatoclax combination activates the intrinsic apoptotic pathway.

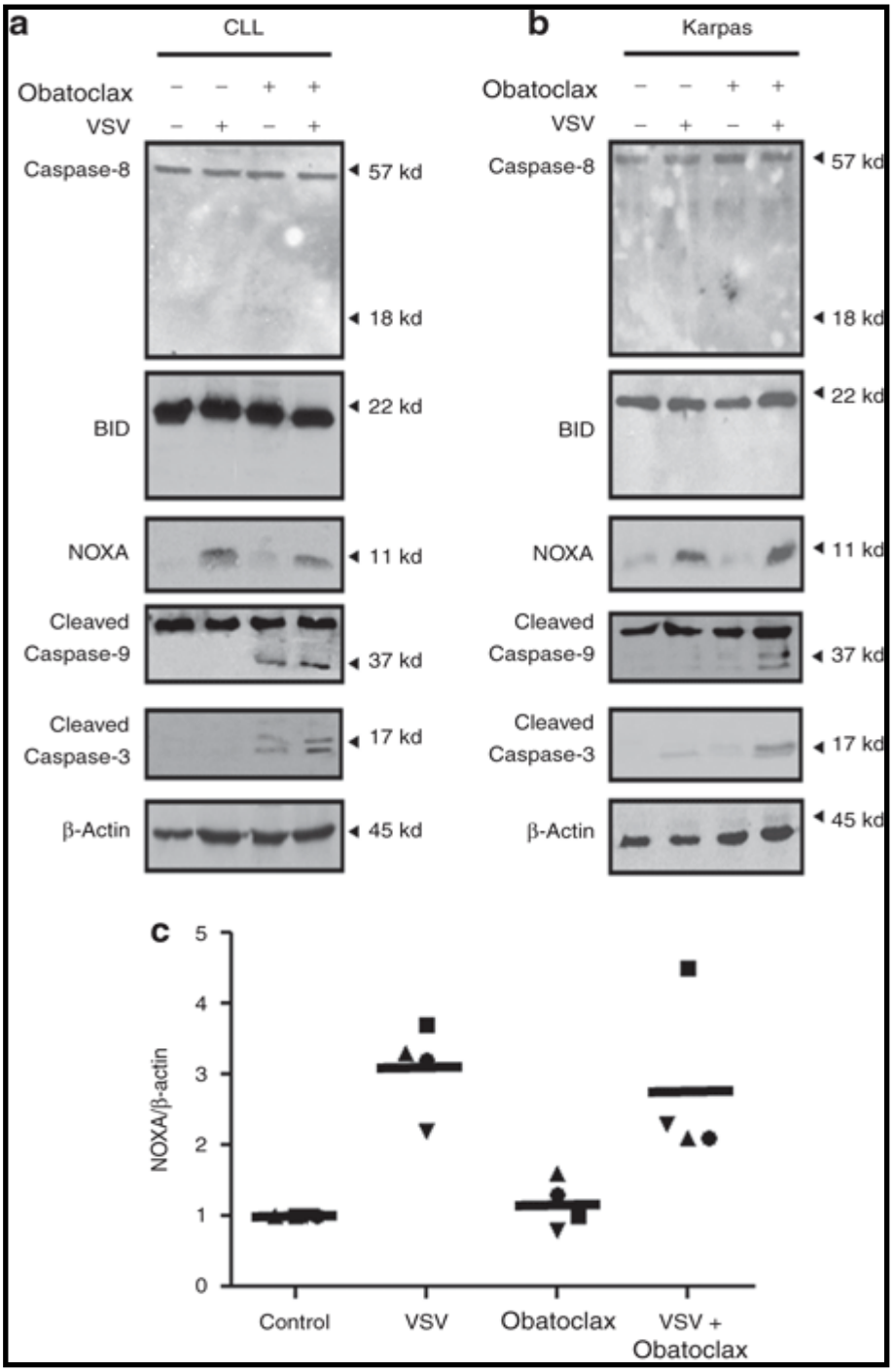

Figure 3 Legend. The effect of VSV and obatoclax single or combination treatments on cleavage of caspase-3, -8, -9 and BID and NOXA expression in (a) PBMCs isolated from CLL patients and (b) Karpas-422 cells was analyzed by immunoblotting. Cells were lysed in 1% CHAPS lysis buffer. Protein lysates were subjected to immunoblot analysis with antibodies that recognize NOXA, cleaved caspase-3 and both cleaved and uncleaved forms of caspase-8, -9, and BID. The CLL patient blots were performed in n = 3 patients and a representative CLL patient is shown. (c) Primary PBMCs isolated from CLL patients were pretreated in the presence or absence of VSV-obatoclax. NOXA and β-actin mRNA levels were determined by real-time PCR. The scatter graphic shows level of NOXA mRNA expression in PBMCs from CLL patients; the line demonstrates the mean value for all patients (n = 4). CLL, chronic lymphocytic leukemia; PBMC, peripheral blood mononuclear cell; VSV, vesicular stomatitis virus.

NOXA induction is essential for obatoclax-mediated apoptosis in Karpas-422 cells. Following VSV infection, the BH-3 only proapoptotic protein NOXA is transcriptionally induced in an interferon regulatory factor-3 and p53-dependent manner.[25,26,27] To determine whether NOXA contributes to VSV-induced apoptosis in CLL, NOXA expression was measured in CLL and Karpas-422 cells. VSV infection alone or in combination with obatoclax-induced NOXA expression equivalently at the RNA and protein levels (Figure 3a–c). Whereas VSV infection did not to trigger caspase-3 cleavage (Figure 3a,b) or loss of cell viability (Figure 1b), the combination resulted in caspase-3 cleavage and decreased cell viability. Silencing of NOXA by introduction of small-interfering RNA (siRNA) resulted in reduced caspase-3 cleavage in cells treated with VSV-obatoclax (Figure 4a). Furthermore, siRNA knockdown of NOXA impaired the VSV-obatoclax-induced apoptotic response by 60% in Karpas-422 cells (Figure 4b), indicating an important role for VSV-induced NOXA expression in the synergic effect of VSV-obatoclax.
Furthermore, replacing VSV infection by NOXA overexpression demonstrated that the combination of NOXA and obatoclax is able to induce caspase-3 cleavage (Figure 4c, lanes 4 and 6) and cell killing (Figure 4d) to levels comparable to VSV-obatoclax combination therapy (Figure 4c, lanes 8 and Figure 4d), suggesting that NOXA is sufficient for induction of apoptosis.

Figure 4: Induction of NOXA expression is necessary and sufficient to synergistically induce apoptosis with obatoclax.

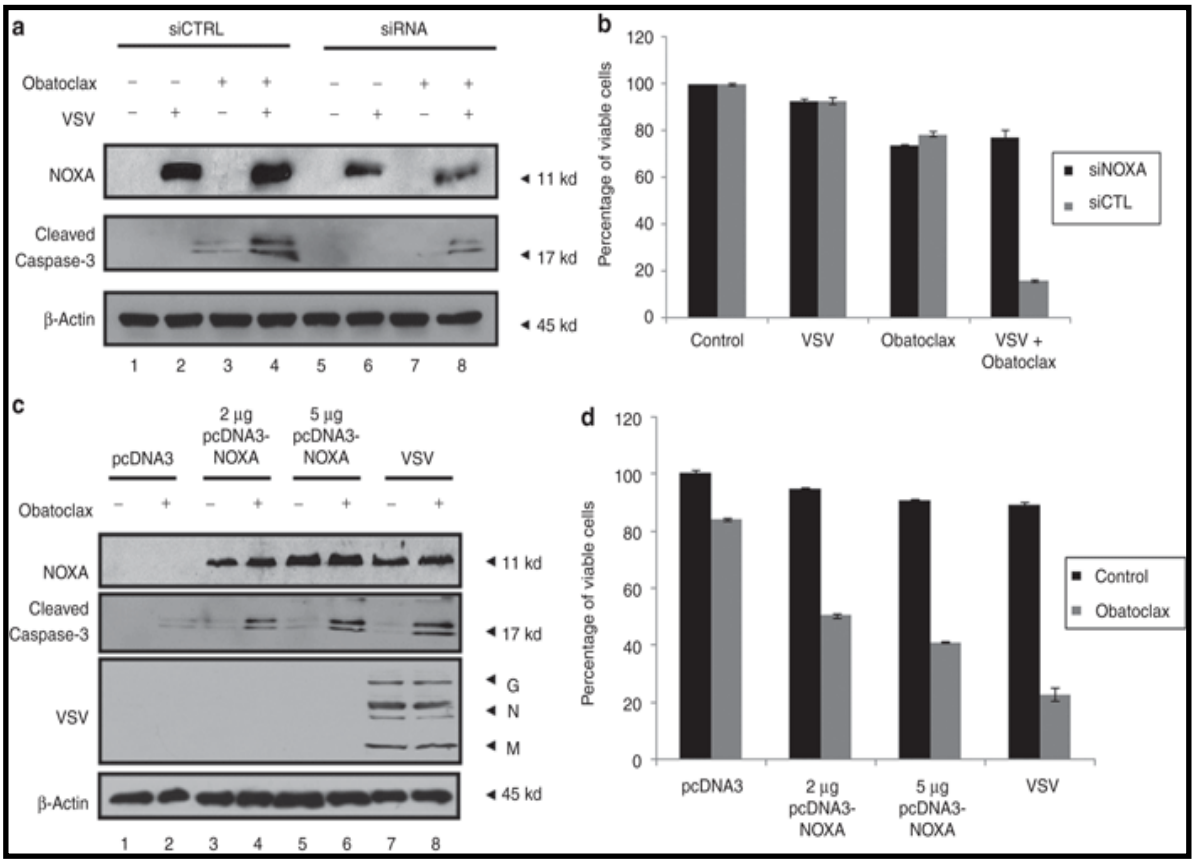

Figure 4 Legend. (a–b) Karpas-422 B-lymphoma cells were transiently transfected with siRNA targeting human NOXA (siNOXA) or the nontargeting control pool (siControl). At 24 hours post-transfection, cells were treated with obatoclax followed or not by VSV infection. (a) At 24 hours postinfection, cells were lysed and NOXA silencing was analyzed by immunoblot using anti-NOXA antibody. Caspase-3 cleavage was also determined using an anti-caspase-3 antibody. (b) Cell viability analysis by Annexin V/PI staining was performed on cells treated as described in a. Black bars represent Karpas-422 cells treated with siNOXA and gray bars represent cells treated with siControl. The data shown are the mean ± SEM (n = 3). (c–d) Karpas-422 B-lymphoma cells were transiently transfected with human pcDNA3-NOXA or empty vector. At 24 hours post-transfection, cells were treated with obatoclax (a). At 24 hours post-treatment, cells were lysed and NOXA expression, caspase-3 cleavage and VSV replication were analyzed by immunoblot. G, glycoprotein; M, matrix; N, nucleocapsid. (b) Cell viability was determined by FACS analysis after Annexin V/PI staining. (d) Black bars represent nontreated Karpas-422 cells and gray bars represent cells treated with obatoclax. The data shown are the mean ± SEM (n = 3). FACS, fluorescence-activated cell sorting; VSV, vesicular stomatitis virus.

VSV-obatoclax combination triggers BAX translocation and cytochrome c release. To examine further the effect of the VSV-obatoclax combination on the stimulation of the intrinsic apoptotic pathway, BAX translocation, oligomerization—determined by the detection of the activated form of BAX with the conformation-specific antibody 6A7—cytochrome c release and NOXA expression in the mitochondria were examined. Treatment with VSV or obatoclax alone did not induce BAX translocation, activation, or cytochrome c release (Figure 5, lanes 2, 3, 6, and 7). VSV-obatoclax combination treatment resulted in recruitment and activation of BAX at the mitochondrial membrane (Figure 5, lane 4) and cytochrome c release into the cytoplasm (Figure 5, lane 8). NOXA protein expression was induced and localized to the mitochondrial fraction in VSV- and VSV-obatoclax-treated cells (Figure 5, lanes 2 and 4). Although VSV alone induced NOXA expression, it was not sufficient to trigger apoptosis and only the VSV-obatoclax combination induced the translocation of activated BAX to the mitochondria and cytochrome c release. Further analysis of BCL-2 family proteins demonstrated that the levels of MCL-1, BAK, and BCL-2 were not altered by VSV or obatoclax single or combination treatments (data not shown).

Figure 5: VSV-obatoclax induces BAX translocation to the mitochondria and cytochrome c release into the cytoplasm.

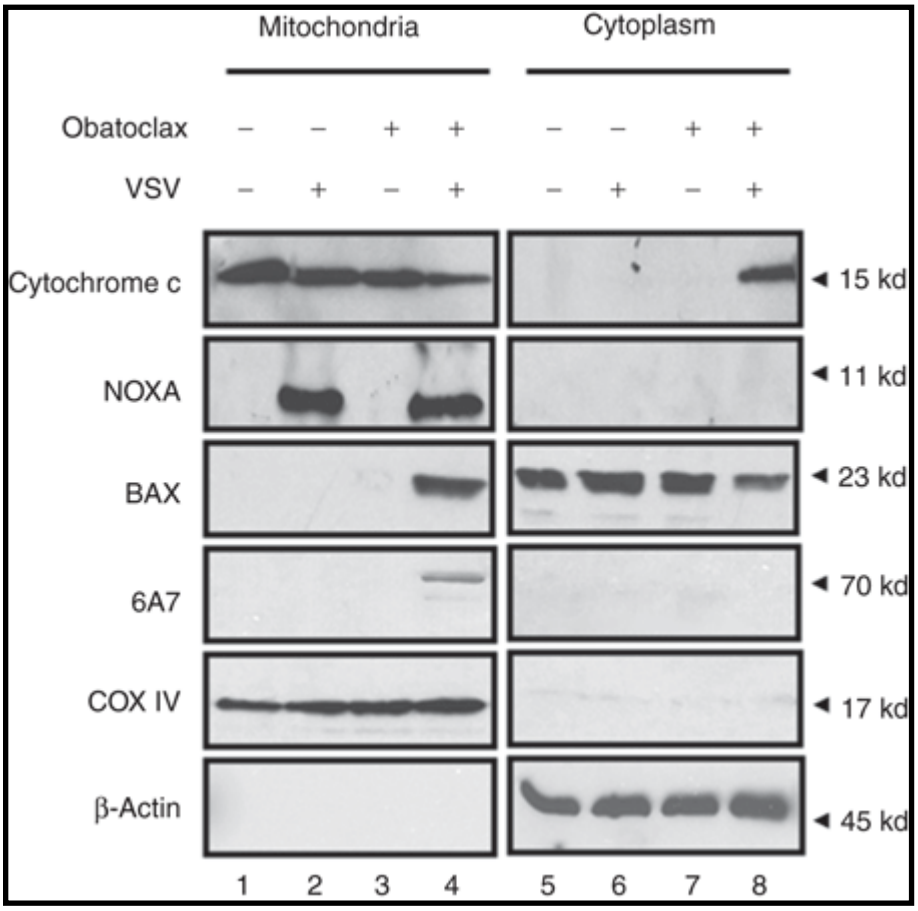

Figure 5 Legend. Following 24 hours, VSV-obatoclax treatment Karpas-422 cells were harvested and mitochondria were isolated using the Pierce mitochondria isolation kit, reagent-based method. The mitochondria-free cytosolic fraction was analyzed for cytochrome c release by immunoblotting using specific antibodies for cytochrome c. The mitochondrial fraction was analyzed for NOXA expression, BAX 6A7 oligomerization and BAX translocation using anti-NOXA, -6A7 and -BAX antibodies. COX IV and β-actin were used as a loading control for mitochondrial and cytosolic fractions, respectively. VSV, vesicular stomatitis virus.

BCL-2 is overexpressed in primary cells from CLL patients. It was previously shown that overexpression of different BCL-2 family members—predominantly BCL-2 and MCL-1—correlate with poor prognosis, disease progression, and resistance to treatment in CLL patients.[5,6,7] Immunoblot analysis showed that BCL-2 was overexpressed in PBMCs isolated from CLL patients, compared with PBMCs from healthy volunteers (Figure 6a). Karpas-422 and Granta-519 expressed BCL-2 levels similar to those observed in CLL patients, as previously shown,[23] whereas other B-cell lines (L428, CRO) displayed lower levels of protein (Figure 6), similar to healthy controls. MCL-1 was not consistently overexpressed in primary CLL cells and levels differed between CLL patients and healthy donors (Figure 6a); however, no fold change could be assessed between the two groups (Figure 6b). These findings suggest that specific targeting of overexpressed BCL-2 by obatoclax in part relieves the inhibition to apoptosis.

Figure 6: Expression of antiapoptotic BCL-2 family protein.

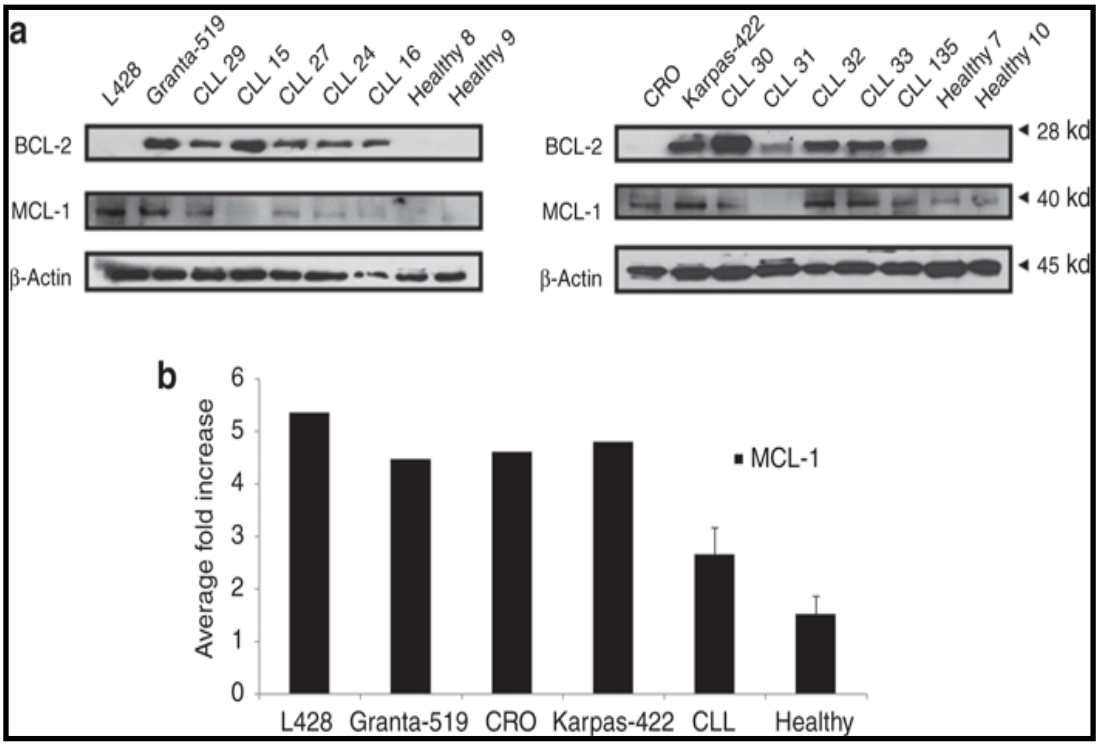

Figure 6 Legend. Expression of antiapoptotic BCL-2 family proteins. (a) Cells from four different B-lymphoma cell lines (L428, Granta-519, CRO, and Karpas-422), primary CLL cells and PBMCs from healthy donors were examined by immunoblotting for BCL-2 and MCL-1 expression. Protein lysate was isolated using 1% CHAPS buffer and BCL-2 and MCL-1 proteins were analyzed by western blot with anti-BCL-2 and MCL-1 antibodies. β-Actin was evaluated as a loading control. (b) The ratio of MCL-1 protein (black bars) in B-lymphoma lines and CLL patients (n = 12) was compared to MCL-1 levels in healthy volunteers (n = 4), respectively. Protein expression levels were quantified and normalized to β-actin level. The data shown are the mean ± SEM. BCL, B-cell lymphoma-2; CLL, chronic lymphocytic leukemia; PBMC, peripheral blood mononuclear cell; MCL-1, myeloid cell leukemia-1.

Combination treatment abrogates BCL-2/BAX and MCL-1/BAK interactions and promotes NOXA/BAX complexes. To examine interactions between pro- and antiapoptotic proteins following the VSV-obatoclax treatment, coimmunoprecipitation experiments were performed in Karpas-422 cells. MCL-1 and BAK interacted in nontreated cells (Figure 7a, lane 1) and this complex was only slightly disrupted with VSV or obatoclax (Figure 7a, lanes 2–3), but was insufficient to induce apoptosis (Figure 5a, lane 3). VSV-obatoclax however caused complete loss of the MCL-1/BAK complexes (Figure 7a). Similarly, BCL-2 and BAX were constitutively present as a heterodimeric complex in untreated cells (Figure 7b, lane 1) and VSV or obatoclax alone had minimal effect on heterodimer formation (Figure 7b, lanes 2 and 3); clearly, the VSV-obatoclax combination caused almost complete dissociation of the BCL-2/BAX complex (Figure 7b, lane 4). In terms of NOXA-containing complexes, VSV-induced NOXA interacted with its cognate binding partner MCL-1 (Figure 7c, lane 2), whereas obatoclax alone or together with VSV disrupted NOXA/MCL-1 interactions (Figure 7c, lanes 3 and 4). The induction of NOXA, as well as the disruption of BCL-2–BAX interaction, suggested that NOXA and BAX may interact to form heterodimers. Coimmunoprecipitation with anti-BAX followed by immunoblot with anti-NOXA confirmed that NOXA interacted with BAX in VSV-infected cells (Figure 7d, lane 2) and VSV-obatoclax increased this interaction by threefold (Figure 7d, lane 4). The reciprocal immunoprecipitation confirmed the identity of this novel NOXA-BAX heterodimer (Figure 7e). Altogether, these experiments argue that the VSV-obatoclax combination shifts the balance of BCL-2 family complexes toward those heterodimers that stimulate mitochondrial-dependent apoptosis in CLL cells.

Figure 7: Combination treatment disrupts BCL-2/BAX and MCL-1/BAK interactions and promotes NOXA/BAX heterodimer formation.

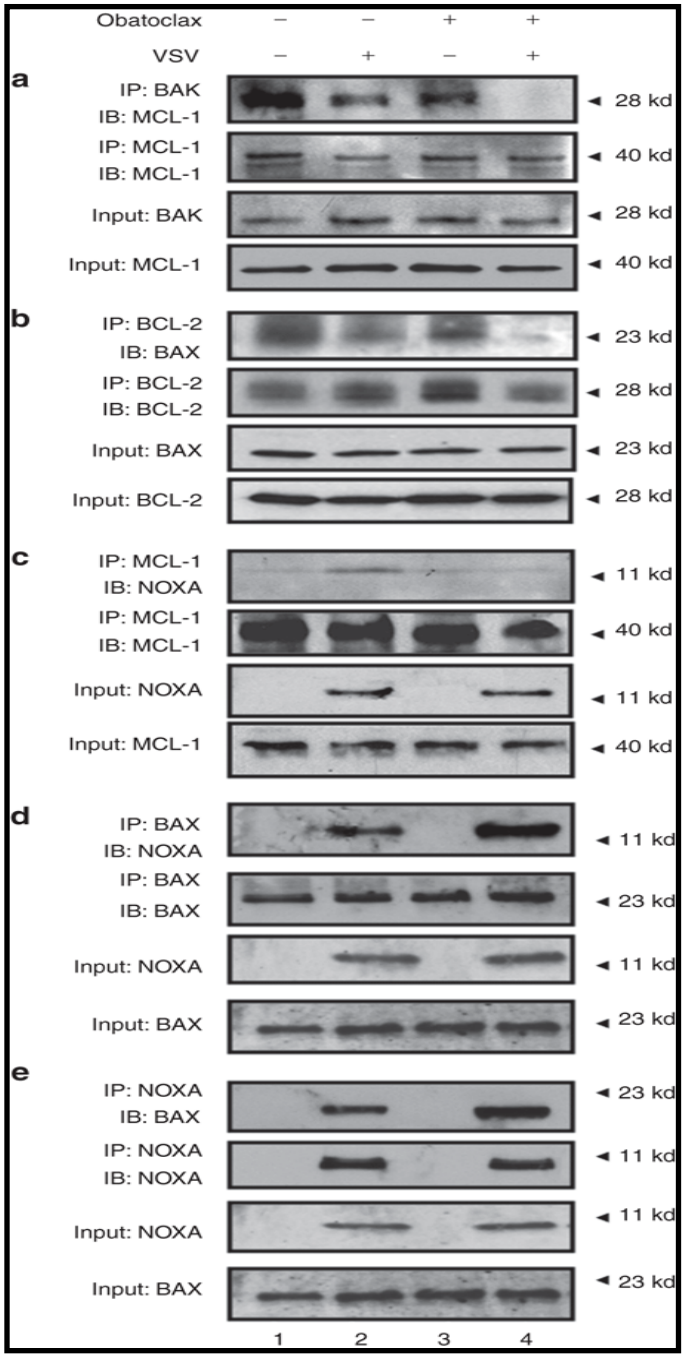

Figure 7 Legend. (a–e) Karpas-422 cells were treated with obatoclax (100 nmol/l) and VSV (10 MOI) for 24 hours. Protein lysates were prepared using 1% CHAPS buffer. (a) MCL-1 was immunoprecipitated (IP) from protein lysate and BAK interaction was revealed by immunoblotting using anti-BAK antibody. Protein inputs for BAK and MCL-1 are shown as separate bands at the bottom of the panel. (b) BCL-2 protein was immunoprecipitated with anti-BCL-2 antibody and coimmunoprecipitated proteins were detected using anti-BAX specific antibody. Protein inputs for BAX and BCL-2 are shown as separate bands at the bottom of the panel. (c) MCL-1 protein was immunoprecipitated with MCL-1 mAbs. MCL-1 immunoprecipitation was performed and bound fractions were analyzed by western blot for NOXA protein. Proteins inputs for NOXA and MCL-1 are shown as separate bands at the bottom of the panel. (d) BAX protein was immunoprecipitated, and coimmunoprecipitated NOXA protein was detected by western blot using a specific antibody for NOXA. Proteins inputs for NOXA and BAX are shown as separate bands at the bottom of the panel. (e) The reverse coimmunoprecipitation was performed in VSV- and obatoclax-treated cells. NOXA protein was immunoprecipitated with NOXA mAbs. Coimmunoprecipitated proteins were detected by immunoblot using specific antibody for BAX. Proteins inputs for NOXA and BAX are shown as separate bands at the bottom of the panel. BCL, B-cell lymphoma-2; mAb, monoclonal antibody; MCL, myeloid cell leukemia-1; MOI, multiplicity of infection; VSV, vesicular stomatitis virus.

Discussion

The objective of the present study was to investigate the molecular mechanisms involved in VSV-obatoclax-mediated apoptotic synergism in CLL. We demonstrate: (i) enhanced cell killing in CLL cell lines, in primary CD5+ CD19+ CLL cells ex vivo, and in a murine model of lymphoma with the VSV-obatoclax combination; (ii) activation of the intrinsic apoptotic pathway, involving VSV-induced NOXA expression, BAX activation and translocation to the mitochondria and cytochrome c release; and (iii) mechanistically, disruption of BCL-2/BAX and MCL-1/BAK complexes and formation of proapoptotic complexes, including a novel NOXA/BAX heterodimer.

There are several historical cases where patients with disseminated cancers have displayed improved conditions following viral vaccination.[33] Oncolytic virotherapy has emerged as an effective treatment for such cancers.[22,34] We and various groups have highlighted the ability of VSV to treat disseminated cancers such as CLL,[23] adult T-cell leukemia[25] and multiple myeloma[22] in preclinical models. Intravenous administration of the virus has been shown to be a successful method of therapy in various animal models and may translate well clinically for the treatment of CLL,[36,37] particularly at the stage where other treatments for advanced disease have failed. It is well understood that disseminated hematological malignancies will be difficult to treat and may require modified methods of therapy such as repetitive and carrier-cell based delivery of the virus.[34,38]

CLL is characterized by overexpression of antiapoptotic proteins such as BCL-2 and MCL-1.[3,4] BCL-2 overexpression is a hallmark of this disease and is found in most cases;[5] interestingly, high levels of MCL-1 expression strongly correlate with aggressive disease, negative clinical outcome and resistance to various treatments and is observed in ~30% of patients.[5,6,7] Under conditions where MCL-1 levels are high proapoptotic BAK can be sequestered by MCL-1 causing resistance to various therapies.[39,40] Although we identified BCL-2 overexpression in PBMCs from CLL

patients and in Karpas-422 cells, MCL-1 levels on average were similar in CLL patients and healthy donors.

The effect of obatoclax on the disruption of MCL-1/BAK complexes has been characterized previously[21,39] but only one group investigated the effect of obatoclax on MCL-1/BAK interactions in CLL;[41] in this case, treatment was used in combination with bortezomib, a proteasome inhibitor that leads to the accumulation of MCL-1 in CLL cells,[42] thus promoting MCL-1/BAK interaction and apoptotic resistance. Although MCL-1 overexpression was not observed in the CLL samples, Karpas-422 had elevated levels of MCL-1 and MCL-1/BAK complexes in resting cells. NOXA binds and inactivates MCL-1 protein, allowing the release and activation of BAK40 and this interaction has been characterized in primary CLL following induction of NOXA.[39] Furthermore, NOXA induction has been observed in primary cells from CLL patients following treatment with histone deacetylase inhibitors[39] and aspirin[43] and was involved in regulating apoptosis and cell survival. The present study demonstrates that disruption of MCL-1/BAK complexes is the result of upregulation of NOXA by VSV and targeting of MCL-1 by obatoclax.

BH-3-only proteins are divisible into two groups: "sensitizers/depressors" that selectively bind antiapoptotic proteins, and "direct activators" that bind to antiapoptotic proteins and proapoptotic BAX and BAK.[8,44] Binding of "direct activators" BID and BIM to BAX and BAK has been shown to trigger conformational change and activation of BAX/BAK followed by mitochondrial outer membrane permeabilization.[8,45,46] NOXA is considered a "sensitizer/depressor" BH-3-only protein,[8,44] although a previous report suggested interaction between NOXA and BAX following double-stranded RNA or virus infection.[27] The present study is the first to identify endogenous NOXA-BAX heterodimers; NOXA is present at the mitochondria, as is activated BAX, suggesting that NOXA/BAX interactions at the mitochondrial surface contribute to membrane permeabilization, cytochrome c release, and apoptosis (Figure 8), Combination treatment is likely to affect other BCL-2 family members; previous studies demonstrated that obatoclax can induce BIM expression and that inhibition of MCL-1 can alter the BAK activity, as well as BAX.[16,17,21] Additionally, other BCL-2 proteins may form apoptosis-inducing

complexes that were not detected in the present study. Moreover, other groups have reported that activation of the JAK/STAT pathway can induce apoptosis in CLL cells.[47] Further studies will clarify the role of these additional proteins in VSV-obatoclax synergism.

Synergism between VSV and obatoclax appears to require three major events: NOXA upregulation, BAX release from BCL-2 and BAK release from MCL-1. The importance of NOXA in apoptotic induction[25,26,27] was highlighted by the observation that siRNA-mediated knockdown of NOXA decreased apoptosis in VSV-obatoclax treated cells and that VSV-mediated NOXA induction can be replaced with NOXA expression plasmids to synergistically induce apoptosis with obatoclax. VSV alone did not cause significant CLL cell death (Figure 3a, lanes 1–3), likely due to BAX sequestration by overexpressed BCL-2.11 Although, NOXA induction by VSV was accompanied by a small increase in NOXA/MCL-1 interactions and a small decrease in MCL-1/BAK interactions, it was insufficient to shift the balance from prosurvival to proapoptotic complexes; only in combination with obatoclax did VSV completely relieve BCL-2 and MCL-1-mediated inhibition of BAX and BAK, respectively. These results strongly suggest NOXA expression is an important element in VSV-obatoclax-mediated cell death. In summary, we propose VSV-obatoclax therapy in primary CLL cells induces apoptosis via a mitochondrial-dependent pathway in which cytochrome c release and activation of caspases-9 and -3 are triggered by VSV-induced NOXA expression and obatoclax-induced release of BAX and BAK (Figure 8).

Figure 8: A proposed model of VSV-obatoclax synergism.

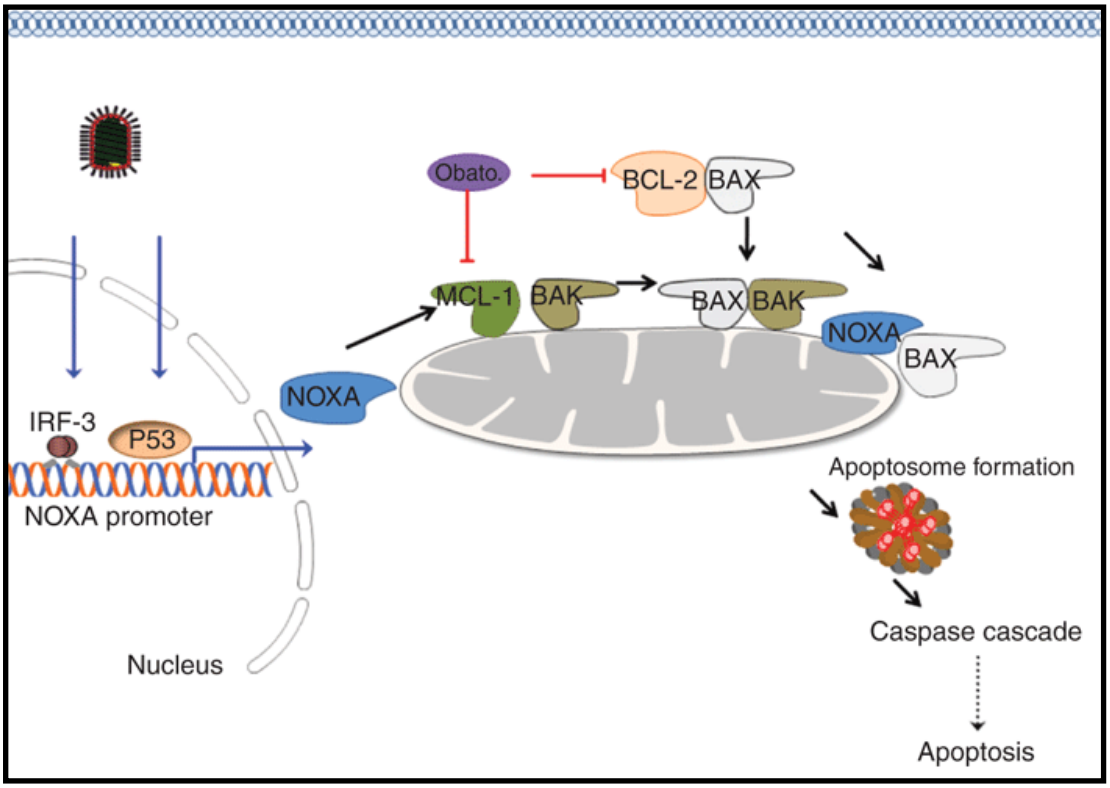

Figure 8. Legend. In CLL cells, the proapoptotic activity of BAX and BAK are inhibited via association with antiapoptotic BCL-2 and MCL-1, respectively. Obatoclax (GX15-070) occupies the BH-3-binding groove of prosurvival BCL-2 and MCL-1 to disrupt BCL-2/BAX and MCL-1/BAK heterodimers, thus restoring the ability of the intrinsic apoptotic pathway to respond to VSV apoptotic stimuli. In response to VSV infection, the BH-3-only protein NOXA is induced at the transcriptional level, additionally contributing to the abrogation of MCL-1/BAK complexes. Activated NOXA protein may also directly induce a conformational change in BAX that subsequently promotes translocation to the mitochondria outer membrane. The VSV-obatoclax combination therapy leads to the activation and oligomerization of BAX and BAK homo- and heterodimers and thus membrane permeabilization, cytochrome c formation of the apoptosome and activation of the caspase cascade. BCL, B-cell lymphoma-2; CLL, chronic lymphocytic leukemia; MCL, myeloid cell leukemia-1; Obato., obatoclax; VSV, vesicular stomatitis virus.

Acknowledgements

We thank GeminX for the generous gift of obatoclax. We also thank Gordon Shore and Mai Nguyen (McGill University) for pcDNA3-NOXA plasmid and reagents. This research was supported by grants to J.H. from the National Cancer Institute of Canada (NCIC) with funds from the Terry Fox Foundation and the Canadian Institutes of Health Research. V.F.T. was supported by a Studentship from NSERC, S.O. by a Studentship from FRSQ, T.L.-A.N. by a Fellowship from FRSQ.

Material and Methods

Patients and PBMC isolation. PBMCs were obtained from healthy individuals and CLL patients at the Jewish General Hospital (Montreal, QC, Canada) following written informed consent, in agreement with the Jewish General Hospital and McGill University Research Ethics Committee. Patients had a median age of [60]. Samples were collected from both male and female patients, although a majority were from male donors. The absolute lymphocyte counts were typical of CLL patients in general. Patients were not receiving treatment at the time of sample collection. PBMCs were isolated as previously described.[23] PBMCs were cultured in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (Wisent, St-Bruno, Quebec, Canada) and 100 U/ml penicillin–streptomycin. PBMCs were cultured at 37 °C in a humidified, 5% CO2 incubator. CLL was confirmed by presence of CD5+ and CD19+ markers. Only patients with a 30% or greater CD5+/CD19+ CLL cell population were used in this study.[48,49]

Cell lines. The human B-lymphoma cell line Karpas-422 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the A20 mouse B-lymphoma cell line was purchased from ATCC (Manassas, VA). All cell lines were grown in RPMI 1640 medium (Wisent, St Bruno, QC, Canada) supplemented with 10% fetal calf serum, penicillin and streptomycin. Cells were maintained at 37 °C and 5% CO_2.

Virus production, quantification, and infection. Construction of VSV-AV1 was previously described.[36] Virus stock was grown in Vero cells (purchased from ATCC, Bethesda, MD), concentrated from cell-free supernatants by centrifugation (15,000 r.p.m./4 °C/90 minutes) and titrated in duplicate by standard plaque assay as previously described.[23] Primary PBMC isolates and Karpas-422 cells were infected with VSV at a multiplicity of infection of 10 plaque-forming units/cell for 1 hour in serum-free media at 37 °C. The cells were then incubated with complete medium at 37 °C for the indicated times.

Viability assay. Cell viability was assessed by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium dye absorbance according to the manufacturer's instructions (Chemicon, Billerica, MA). PBMCs were seeded in 96-well plates at a density of 5 × 105 cells/well. Cell viability was also analyzed by Annexin V and propidium iodide staining and fluorescence-activated cell sorting analysis. For drug combination studies, cells were incubated with or without obatoclax (100 nmol/l) and infected or not with VSV-AV1 (10 multiplicity of infection) as indicated. To determine the IC50, increasing concentrations of obatoclax (0–20 μmol/l) were used. Plates were incubated at 37 °C, 5% CO2 and cells analyzed every 24 hours for 7 days. Each experimental condition was performed in quadruplicate.

Protein extraction and western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline, and proteins were extracted as described previously.23 Briefly, cell pellets were lysed in ice-cold buffer containing phosphate-buffered saline, 0.05% NP40, 0.1% glycerol, 30 mmol/l NaF, 40 mmol/l β-glycerophosphate, 10 mmol/l Na3VO4, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) in 1/1,000 dilution. Extracts were kept on ice for 15 minutes and centrifuged at 10,000 g for 25 minutes (4 °C), and supernatants were stored at −80 °C. Protein concentration was determined with Bio-Rad protein assay reagent (BioRad, Hercules, CA). Protein extracts were resolved using 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Hybond C Super; GE Healthcare Bio-Sciences, Buckinghamshire, UK). Membranes were blocked for 1 hour in 5% nonfat dried milk in TBST (Tris-buffered saline + 0.5% Tween-20). Followed by incubation with any of the following primary antibodies: cleaved caspase-3, β-actin, and BID (Cell Signaling Technologies, Danvers, MA; 1:2,000); BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2,000), BAX (Santa Cruz Biotechnology; 1 μg/ml) and anti-mouse NOXA (Calbiochem, San Diego, CA; 1 μg/ml).

Mitochondria isolation. Mitochondria and cytosolic fractions were prepared from Karpas-422 cells after 24 hours of obatoclax and/or VSV-AV1 treatment, using the

Pierce Mitochondria Isolation Kit for cultured cells, reagent-based method according with manufacturer protocol. Fractions were analyzed via western blot for cytochrome c (BD Biosciences, Franklin Lakes, NJ), BAX 6A7, BAX (Santa Cruz Biotechnology) and NOXA (Calbiochem). COXIV is an inner mitochondria membrane protein and was used as a measure of mitochondria purity and loading control; β-actin was used for cytoplasm purity and loading control.

Coimmunoprecipitation of BCL-2 family proteins. Two microgram of anti-BCL-2 monoclonal antibody, 2 μg of anti-BAX monoclonal antibody, 2 μg of anti-MCL-1 (Santa Cruz Biotechnology), 2 μg of anti-BAK monoclonal antibody (Millipore, Temecula, CA) or 2 μg of anti-NOXA monoclonal antibody (Calbiochem) were crosslinked to 20 μg of protein l-agarose beads (Santa Cruz Biotechnology) using 0.2 mol/l triethanloanime pH 8.0. Cells were lysed with 1% CHAPS lysis buffer [10 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 1% CHAPS] containing protease inhibitors and total protein (500 μg) was incubated with crosslinked antibody in 1% CHAPS lysis buffer at 4 °C overnight on a rotator. Immunoprecipitates were collected by centrifugation for 1 minute. The pellets were washed three times with 1% CHAPS lysis buffer, beads were boiled in loading buffer and bound protein was analyzed by western blotting. Samples with antibody alone (no lysate), lysate alone (no antibody) or with an irrelevant isotype-matched immunoglobulin G antibody were used as negative controls (data not shown). Protein input (30 μg) was run in 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Hybond C Super; GE Healthcare Bio-Sciences). Membranes were blocked for 1 hour in 5% nonfat dried milk in TBST (Tris-buffered saline + 0.5% Tween-20) followed by incubation with any of the following primary antibodies: BCL-2, MCL-1 (Santa Cruz Biotechnology; 1:2,000), BAX (Sigma, St Louis, MO; 1 μg/ml), BAK (Millipore and NOXA (Calbiochem; 1 μg/ml).

RNA extraction and real-time PCR. Whole RNA from treated cells was extracted using RNase extraction Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Real time-PCR was performed using 1 μg and 200 ng

(for Karpas-422 and ex vivo CLL cells, respectively) of RNA resuspended in RNase-free ddH2O and Oligo dT12-18 primer (Invitrogen Canada, Burlington, ON, Canada) according to the manufacturer's conditions. Reverse transcription was performed using Superscript II (Invitrogen Canada) at 42 °C for 1 hour. The PCR primer pair specific for NOXA was: forward 5′-AGTAGCTGGAAGTCGAGTGT-3′ and reverse 5′-AGGTTCCTGAGCAGAAGAGT-3′. All data are presented as a relative quantification with efficiency correction based on the relative expression of target genes versus β-actin as reference gene. Complementary DNA was amplified using SyBR Green I PCR master mix (Applied Biosystems, Foster City, CA) and the data was collected using the AB 7500 Real-Time PCR System (Applied Biosystems) and analyzed by Comparative CT Method using the SDS v1.3.1 Relative Quantification Software. For semiquantitative real time-PCR, amplification products were resolved on an agarose gel and digital image of the ethidium bromide stained bands inverted for presentation.

In vivo murine lymphoma model. This study was approved by the local animal care and institutional animal ethics committee of Jewish General Hospital and McGill University. A total of eight animals were used for this study 4–6-week-old female Fox Chase SCID mice (Charles River Laboratories, Pointe Claire, QC, Canada) were injected subcutaneously with 1×10^6 A20 cells in a 100-μl volume into the hind flanks. Tumor volumes were measured and calculated as ½(length × width2).50 Once tumors were palpable, animals were randomly assigned to treatment groups and received five intraperitoneal injections of obatoclax (3 mg/day/kg). At days 2 and 5 following obatoclax injection, VSV-AV1 was inoculated intratumorally at 1×10^8 plaque-forming units of virus each. Animals were evaluated for signs of stress such as infection, dehydration, weight loss (>20%), and limb paralysis.

Transient transfection of siRNA NOXA and NOXA expression plasmid. Control and NOXA-specific RNAi sequences were described previously[26] Transfection of Karpas-422 cells was carried out by electroporation using the Nucleofection System

(Amaxa, Köln, Germany), according to the protocols proposed by the manufacturer. Briefly, 1×10^6 Karpas-422 cells were resuspended in 100 µl of nucleofector V solution (Nucleofector kit V) containing 100 pmol of double-stranded siRNAs. After electroporation (program T020), 500 µl of prewarmed cultured medium were added to the cuvette, and the cells were transferred into cultures plates containing prewarmed culture medium. At the optimal time of gene silencing (24 hours post-transfection), cells were mock-infected, treated or not with 100 nmol/l of obatoclax or infected with VSV 10 multiplicity of infection. After 24 hours cells were collected, protein was extracted, and immunoblots were performed.

Transfection of the pcDNA3-NOXA expression vector was carried out as described above with some changes. Briefly, 1 × 106 Karpas-422 cells were resuspended in 100 µl of nucleofector V solution (Nucleofector kit V) containing increasing amounts of pcDNA3-NOXA complemented with empty vector. Cells were treated with obatoclax 24 hours after transfection. Forty-eight hours post-transfection cells were collected, protein was extracted, and immunoblots were performed.

Statistical analysis. Graphics and statistical analysis were executed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Differences among the treatment groups were analyzed by paired t-test. The P values <0.05 were considered statistically significant. Average values were expressed as mean ± SD.

References

1. Caligaris-Cappio F, T JH. B-cell chronic lymphocytic leukemia: a bird of a different feather. *J Clin Oncol* 1999; **17:** 399-408.

2. Herishanu Y, Polliack A. Chronic lymphocytic leukemia: A review of some new aspects of the biology, factors influencing prognosis and therapeutic options. *Transfusion Apheresis Sci* 2005; **32:** 85-97.

3. Inamdar KV, Bueso-Ramos CE. Pathology of chronic lymphocytic leukemia: an update. *Annals of Diagnostic Pathology* 2007; **11:** 363-89.

4. Craig RW. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. *Leukemia* 2002; **16**(44)**:** 444-54.

5. Pepper C, Lin TT, Pratt G, Hewamana S, Brennan P, Hiller L *et al.* Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers. *Blood* 2008; **112**(9)**:** 3807-17.

6. Johnston JB, Paul JT, Neufeld NJ, Haney N, Kropp DM, Hu X *et al.* Role of myeloid cell factor-1 (Mcl-1) in chronic lymphocytic leukemia. *Leuk Lymphoma* 2004; **45**(10)**:** 2017-27.

7. Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S *et al.* Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with in vitro and in vivo chemoresponses. *Blood* 1998; **91:** 3379-89.

8. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Mol Cell* 2010; **37**(13)**:** 299-310.

9. Kang MH, Reynolds CP. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res* 2009; **15**(4)**:** 1126-32.

10. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci* 2009; **122:** 437-41.

11. Willis SN, Fletcher JI, Kaufmann T, Delft MFv, Chen L, Czabotar PE *et al.* Apoptosis Initiated When BH3 Ligands Engage Multiple Bcl-2 Homologs, Not Bax or Bak. *Science* 2007; **315:** 856-9.

12. Vogler M, Weber K, Dinsdale D, Schmitz I, Schulze-Osthoff K, Dyer M *et al.* Different forms of cell death induced by putative BCL2 inhibitors. *Cell Death Differ* 2009; **16**(7)**:** 1030-9.

13. Campas C, Cosiall AM, Barragan M, Iglesias-Serret D, Santidrian AF, Coll-Mulet L *et al.* Bcl-2 inhibitors induce apoptosis in chronic lymphocytic leukemia cells. *Exp Hematol* 2006; **34:** 1663-69.

14. High LM, Szymanska B, Wilczynska-Kalak U, Barber N, O'Brien R, Khaw SL *et al.* The BH3-mimetic ABT-737 targets the apoptotic machinery in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interactions with established drugs. *Mol Pharmacol* 2010; **77**(3)**:** 483-94.

15. Mason K, Khaw S, Rayeroux K, Chew E, Lee E, Fairlie W *et al.* The BH3 mimetic compound, ABT-737, synergizes with a range of cytotoxic chemotherapy agents in chronic lymphocytic leukemia. *Leukemia* 2009; **23:** 2034-41.

16. Li J, Viallet J, Haura EB. A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. *Cancer Chemother Pharmacol* 2008; **61:** 525-34.

17. Reed JC, Pellecchia M. Apoptosis-based therapies for hematologic malignancies. *Blood* 2005; **106**(2)**:** 408-18.

18. Trudel S, Li Z, Rauw J, Tiedemann R, Wen X, Stewart K. Pre-clinical studies of the pan-Bcl inhibitor obatoclax (GX015-070) in multiple myeloma. *Blood* 2007; **15;109(12):** 5430-8.

19. Witters LM, Witkoski A, Planas-Silva MD, Berger M, Viallet J, Lipton A. Synergistic inhibition of breast cancer cell lines with a dual inhibitor of EGFR-HER-2/neu and a Bcl-2 inhibitor. *Oncology Reports* 2007; **17:** 465-69.

20. Perez-Galan P, Roue G, Villamor N, Campo E, Colomer D. The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood* 2007; **109**(10)**:** 4441-9.

21. Lichty BD, Stojdl DF, Taylor RA, Miller L, Frenkel I, Atkins H *et al.* Vesicular stomatitis virus: a potential therapeutic virus for the treatment of hematologic malignancy. *Hum Gene Ther* 2004; **15**(9)**:** 821-31.

22. Tumilasci VF, Oliere S, Nguyen TL, Shamy A, Bell J, Hiscott J. Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary chronic lymphocytic leukemia cells to vesicular stomatitis virus-induced oncolysis. *J Virol* 2008; **82**(17)**:** 8487-99.

23. Msaouel P, Iankov ID, Allen C, Morris JC, Messling Vv, Cattaneo R *et al.* Engineered measles virus as a novel oncolytic therapy against prostate cancer. *Prostate* 2009; **69**(1)**:** 82-91.

24. Goubau D, Romieu-Mourez R, Solis M, Hernandez E, Mesplede T, Lin R *et al.* Transcriptional re-programming of primary macrophages reveals distinct apoptotic and anti-tumoral functions of IRF-3 and IRF-7. *Eur J Immunol* 2009; **39:** 527-40.

25. Lallemand C, Blanchard B, Palmieri M, Lebon P, May E, Tovey M. Single-stranded RNA viruses inactivate the transcriptional activity of p53 but induce NOXA-dependent apoptosis via post-translational modifications of IRF-1, IRF-3 and CREB. *Oncogene* 2007; **26**(3)**:** 328-38.

26. Sun Y, Leaman DW. Involvement of Noxa in Cellular Apoptotic Responses to Interferon, Double-stranded RNA, and Virus Infection. *J Biol Chem* 2005; **280**(16)**:** 15561-68.

27. Gaddy DF, Lyles DS. Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx. *J Virol* 2007; **81**(6)**:** 2792–2804.

28. Gaddy DF, Lyles DS. Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. *J Virol* 2005; **79**(7)**:** 4170-9.

29. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nature Rev Mol Cell* 2004; **5:** 897-907.

30. Billen L, Shamas-Din A, Andrews D. Bid: a Bax-like BH3 protein. *Oncogene* 2009; **27:** S93-104.

31. Riedl S, Salvesen G. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol.* 2007; **8**(5)**:** 405-13.

32. Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. *Mol Ther* 2007; **15**(4)**:** 651-9.

33. Liu C, Russell SJ, Peng KW. Systemic therapy of disseminated myeloma in passively immunized mice using measles virus-infected cell carriers. *Mol Ther* 2010; **18**(6)**:** 1155-64.

34. Cesaire R, Oliere S, Sharif-Askari E, Loignon M, Lezin A, Olindo S *et al.* Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia. *Oncogene* 2006; **25**(3)**:** 349-58.

35. Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S *et al.* VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 2003; **4**(4)**:** 263-75.

36. Ebert O, Harbaran S, Shinozaki K, Woo SL. Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. *Cancer Gene Ther* 2005; **12**(4)**:** 350-8.

37. Power AT, Wang J, Falls TJ, Paterson JM, Parato KA, Lichty BD *et al.* Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. *Mol. Ther* 2007; **15:** 123-30.

38. Inoue S, Riley J, Gant TW, Dyer MJ, Cohen GM. Apoptosis induced by histone deacetylase inhibitors in leukemic cells is mediated by Bim and Noxa. *Leukemia* 2007; **21**(8)**:** 1773-82.

39. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E, Trichet V, Robillard N, Philippe M *et al.* Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res* 2007; **67**(11)**:** 5418-24.

40. Perez-Galan P, Roue G, Lopez-Guerra M, Nguyen M, Villamor N, Montserrat E *et al.* BCL-2 phosphorylation modulates sensitivity to the BH3 mimetic GX15-070 (Obatoclax) and reduces its synergistic interaction with bortezomib in chronic lymphocytic leukemia cells. *Leukemia* 2008; **22:** 1712-20.

41. Nencioni A, Hua F, Dillon CP, Yokoo R, Scheiermann C, Cardone MH *et al.* Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. *Blood* 2005; **105**(8)**:** 3255-62.

42. Iglesias-Serret D, Piqué M, Barragán M, Cosialls AM, Santidrián AF, González-Gironès DM *et al.* Aspirin induces apoptosis in human leukemia cells independently of NF-kappaB and MAPKs through alteration of the Mcl-1/Noxa balance. *Apoptosis* 2010; **15**(2)**:** 219-29.

43. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR *et al.* BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005; **17:** 525-35.

44. Lovell JF, Billen LP, Bindner S, Shamas-Din A, Fradin C, Leber B *et al.* Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. *Cell* 2008; **135:** 1074-84.

45. Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG *et al.* BAX activation is initiated at a novel interaction site. *Nature* 2008; **455**(23)**:** 1076-82.

46. Nguyen M, Marcellus RC, Roulston A, Watson M, Serfass L, Madiraju SRM *et al.* Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc. Natl. Acad. Sci.* 2007; **104**(9)**:** 19512–17.

47. Liang X, Moseman EA, Farrar MA, Bachanova V, Weisdorf DJ, Blazar BR *et al.* Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood* 2010; **115**(24)**:** 5041-52.

48. Abbott BL. Chronic lymphocytic leukemia: recent advances in diagnosis and treatment. *Oncologist* 2006; **11**(1)**:** 21-30.

49. Abbott BL. Recent advances in chronic lymphocytic leukemia. *Cancer Invest* 2006; **24**(3)**:** 302-9.

50. Jensen MM, Jørgensen JT, Binderup T, Kjær A. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. *BMC Medical Imaging* 2008; **8**(16).

Manuscript II

BCL-2 inhibitors sensitize therapy-resistant chronic lymphocytic leukemia cells to VSV oncolysis

Mol Ther. 2013 May 21. doi: 10.1038/mt.2013.91

Sara Samuel[1], Vladimir Beljanski[2], Julien Van Grevenynghe[1], Stephanie Richards[2], Fethia Ben Yebdri[1], Zhong He[2], Carmen Nichols[2], S.Mehdi Belgnaoui[1], Courtney Steel[2], Marie-Line Goulet[1], April Shamy[3], Dawn Brown[4], Guillermo Abesada[4], Elias K. Haddad[2] and John Hiscott[2*]

[1]*Lady Davis Institute - Jewish General Hospital, McGill University, Montreal, CA*

[2]*Vaccine & Gene Therapy Institute of Florida, Port St. Lucie, FL*

[3]*Dept. of Medicine, Jewish General Hospital, McGill University, Montreal, CA*

[4]*Weissman Oncology Center, Martin Health Systems, Stuart, FL*

Corespondence: Dr. John Hiscott
Division of Infectious Diseases
VGTI Florida
9801 Discovery Way
Port St. Lucie FL 34987
Email: jhiscott@vgtifl.org

Suggested running title: BCL-2 inhibitors and VSV trigger autophagy in CLL

Rationale for Manuscript II

Autophagy is the recycling or degradation of a cell's own components as a survival mechanism. It is also used as a defence mechanism against environmental insult. In several cancer models, Beclin-1, a central autophagy protein, is inhibited by pro-survival apoptotic Bcl-2 proteins to prevent the induction of autophagy and stimulate the development of cancer formation. Bcl-2 family regulation of Beclin-1 promotes crosstalk between the apoptotic and autophagic pathways. In this study, we examined whether the pharmacologic disruption of pro-survival Bcl-2 proteins/Beclin-1 interactions using Bcl-2 inhibitors can stimulate autophagy and sensitize primary CLL cells to the cytotoxic treatments of VSV-AV1. The specific aims of this study were to:

1) Determine whether Bcl-2 inhibitors, Obatolcax, and ABT-737 synergistically induce autophagy in CLL cells when combined with VSV-AV1

2) Evaluate the relative contributions of autophagy and apoptosis to CLL cell killing

3) Characterize the gene expression profiles in CLL patients samples treated with VSV-AV1/BCL-2 inhibitor

Abstract

Many primary cancers including chronic lymphocytic leukemia (CLL) are resistant to Vesicular Stomatitis Virus (VSV)-induced onolysis due to overexpression of the anti-apoptotic and anti-autophagic members of the BCL-2 family. In the present study, we investigated the mechanisms of CLL cell death induced as a consequence of VSV infection in the presence of BCL-2 inhibitors, Obatoclax and ABT-737 in primary ex-vivo CLL patient samples. Microarray analysis of primary CD19+ CD5+ CLL cells treated with Obatoclax and VSV revealed changes in expression of genes regulating apoptosis, the mTOR pathway, and cellular metabolism. A combined therapeutic effect was observed for VSV and BCL-2 inhibitors in cells from untreated patients and from patients unresponsive to standard of care therapy. Additionally, combination treatment induced several markers of autophagy – LC3-II accumulation, p62 degradation and staining of autophagic vacuoles. Inhibition of early stage autophagy using 3-methyladenine led to increased apoptosis in CLL samples. Mechanistically, combination of BCL-2 inhibitors and VSV disrupted inhibitory interactions of Beclin-1 with BCL-2 and MCL-1, thus biasing cells toward autophagy. We propose a mechanism in which changes in cellular metabolism, coupled with pharmacologic disruption of the BCL-2-Beclin1 interactions, facilitate induction of apoptosis and autophagy to mediate the cytolytic effect of VSV.

Introduction

Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia in the Western hemisphere (Chiorazzi *et al.*, 2005; Shanshal *et al.*, 2012), a clonal malignancy characterized by peripheral blood lymphocytosis as a result of defective apoptosis signaling (Shanshal *et al.*, 2012) and the abnormal accumulation of CD5+ monoclonal B lymphocytes (Hallek, Cheson, *et al.*, 2008; Shanshal *et al.*, 2012). Overexpression of anti-apoptotic regulators of the B-cell lymphoma-2 (BCL-2) family contribute to resistance to programmed cell death, drug resistance, disease progression and poor clinical outcome in CLL patients in response to conventional therapies (Buggins *et al.*, 2010). The BCL-2 family is divided into three groups: 1) Anti-apoptotic (BCL-2, myeloid cell leukemia (MCL)-1 and BCL-X_L,), 2) pro-apoptotic (BAX and BAK) and 3) BH3-only (NOXA, PUMA, BID, BIM, BAD, BIK and BMF) proteins (Brunelle *et al.*, 2009; Chipuk *et al.*, 2010; Garcia-Saez, 2012). In addition to their contribution to apoptosis, the BCL-2 family is involved in regulation of autophagy, a cellular process characterized by the sequestration of cytoplasmic material into vacuoles for bulk degradation (Levine *et al.*, 2008; Pattingre *et al.*, 2006; Pattingre *et al.*, 2005). Beclin-1 is a BH3-only protein, a central autophagy regulator and a haploinsufficient tumor suppressor that is inhibited by anti-apoptotic BCL-2 and BCL2-X_L proteins; this interaction blocks autophagy progression in cancer cells (Pattingre *et al.*, 2006; Pattingre *et al.*, 2005; Sinha *et al.*, 2008) and serves as a regulatory point of crosstalk between the apoptotic and autophagic pathways (Gordy *et al.*, 2012; Hou *et al.*, 2010; Zhou *et al.*, 2011). Several other proteins also negatively regulate autophagy, including mechanistic target of rapamycin (mTOR), a Ser/Thr protein kinase involved in growth, proliferation and cell cycle progression (Townsend *et al.*, 2012).

Oncolytic virotherapy is currently being tested with promising results in phase I-III clinical trials (Breitbach *et al.*, 2011; Donnelly *et al.*, 2012; Eager *et al.*, 2011; Rowan, 2010; Russell *et al.*, 2012). Vesicular Stomatitis Virus (VSV) has emerged

as a prototypical oncolytic virus (OV) that induces direct tumor cell lysis, is sensitive to type I interferon (IFN)-induction and cellular antiviral responses (Barber, 2004; Lichty, Power*, et al.*, 2004; Lichty, Stojdl*, et al.*, 2004; Stojdl *et al.*, 2003) and activates intrinsic and extrinsic apoptotic signaling (Gaddy *et al.*, 2007; Samuel *et al.*, 2010; Tumilasci *et al.*, 2008). VSV used in this study has a M51R substitution in the viral matrix (M) protein that was shown to enhance the safety profile of the virus; the attenuated mutant is a potent inducer of the IFN response in healthy cells (Barber, 2004; Stojdl *et al.*, 2003).

BCL-2 inhibitors (BH3 mimetics) represent a new class of anti-cancer therapeutics that display promising results in pre-clinical and clinical studies when used as single agents or in combination with conventional cancer therapies (Campas *et al.*, 2006; High *et al.*, 2010; Mason *et al.*, 2009). Functionally, this class of inhibitors competes with BH3-only pro-apoptotic proteins for binding to anti-apoptotic BCL-2 proteins (Bajwa *et al.*, 2012; Oltersdorf *et al.*, 2005). Obatoclax (GX15-070) is a pan BCL-2 inhibitor and a synthetic derivative of prodiginine (Nguyen *et al.*, 2007; Pandey *et al.*, 2009) . ABT-737 and its orally active analogue ABT-263 (Navitoclax) are BAD-like mimetics; the ABT compounds target the majority of the anti-apoptotic BCL-2 proteins but have low affinity for MCL-1 (Oltersdorf *et al.*, 2005; Tse *et al.*, 2008). Obatoclax and ABT-263 are currently in multiple phase I/II clinical trials for the treatment of various solid and hematological malignancies including CLL, non-Hodgkin's lymphoma and lung cancer (Gandhi *et al.*, 2011; Paik *et al.*, 2010; Roberts *et al.*, 2012).

We previously reported that Obatocalx displaced pro-survival interactions, while VSV infection both stimulated expression of the BH3-only NOXA in an IRF-3 dependent manner and the formation of a NOXA-BAX pro-apoptotic complex (Samuel *et al.*, 2010). With a growing interest in pharmacological disruption of anti-apoptotic and anti-autophagic interactions in CLL treatments, we expanded our previous studies to also examine a novel, more specific BCL-2 inhibitor ABT-737, performed microarray analysis of both leukemic and non-leukemic cells exposed to VSV + Obatoclax, and examined contribution of an alternative cell death pathway

(autophagy) to CLL cell death. Gene expression profiling of CLL patient samples treated with BCL-2 inhibitor Obatoclax, VSV, or the combination was performed to gain insight into mechanisms that are responsible for combined therapeutic effects by examining changes in expression profiles; this analysis revealed changes in expression of genes regulating apoptosis, the mTOR pathway, and cellular metabolism. Combination therapy in primary CLL cells *ex vivo* selectively targeted CD19+ CD5+ leukemic cells from both untreated patients and patients resistant to standard of care therapy. Mechanistically, VSV and ABT-737 treatment induced both apoptosis and autophagy in CLL cells by disrupting the inhibitory interactions of Beclin1 with BCL-2 and MCL-1, thus biasing cells toward autophagy and apoptotic cell death.

Results

Molecular and clinical features of the CLL patients. Several clinical prognostic and biological characteristics (Cramer *et al.*, 2011; Hallek, Cheson*, et al.*, 2008; Shanshal *et al.*, 2012) of CLL patients included in this study are summarized in **Table 1**. Elevated Zap70 and CD38 expression, p53 mutation, chromosomal abnormalities (Fluorescence In Situ Hybridization (FISH)) and unmutated IgVH all have prognostic significance and have been associated with an unfavorable clinical course and resistance to traditional therapy. Patients examined in the study were grouped as: 1) previously untreated; 2) non-responders/refractory; or 3) responders to chemotherapy and/or antibody-based therapies. Given that CLL is characterized by the progressive accumulation of small mature CD5+ B lymphocytes (Shanshal *et al.*, 2012), we measured by flow cytometry, the CD19 and CD5 B cell subsets in CLL patients and healthy donors as listed in **Table 1**. The percentage of leukemic CD19+ CD5+ B-cells was dramatically higher in untreated (44.4%) and refractory (59.4%) representative CLL patients, compared to responders (2.2%) or healthy controls (0.5%) **(Fig 1a-d)**. The total percentage of cells with CD19+ CD5+ and CD19+ CD5- marker expression for all the CLL patients and healthy donors included in this study are listed in **Fig 1e.**

Table 1: Patient Characteristics.

Characteristics	(*n*)
CD5	
Positive (>30%)	11
Negative (<30%)	11
CD38	
Positive (>30%)	5
Negative (<30%)	7
Not assessed	10
Current disease status	
Untreated	13
Responder	5
Non-responder/refractory	4
p53 Mutation	
Mutated	0
Non-mutated	2
Not assessed	20
FISH analysis	
No deletion	3
Trisomy 12	1
Trisomy 12/14	1
13q deletion	1
11q22 deletion	1
17p13 deletion	1
12, 17p deletion	1
20q deletion	1
Not assessed	11
IgVH status	
Mutated	1
Not mutated	5
Not assessed	16
ZAP70	
Positive	4
Negative	2
Not assessed	16
Age, years (median range)	67
Gender	
Female	3
Male	19
Total	22

Abbreviations: CD, cluster of differentiation, FISH, fluorescence *in situ* hybridization; IgVH, immunoglobulin heavy chain variable gene; *n*, number of patients with particular characteristic; p53, tumor protein 53; ZAP70, 70-kDa zeta chain-associated protein kinase.

Figure 1: Distribution of lymphocyte subsets in CLL patients

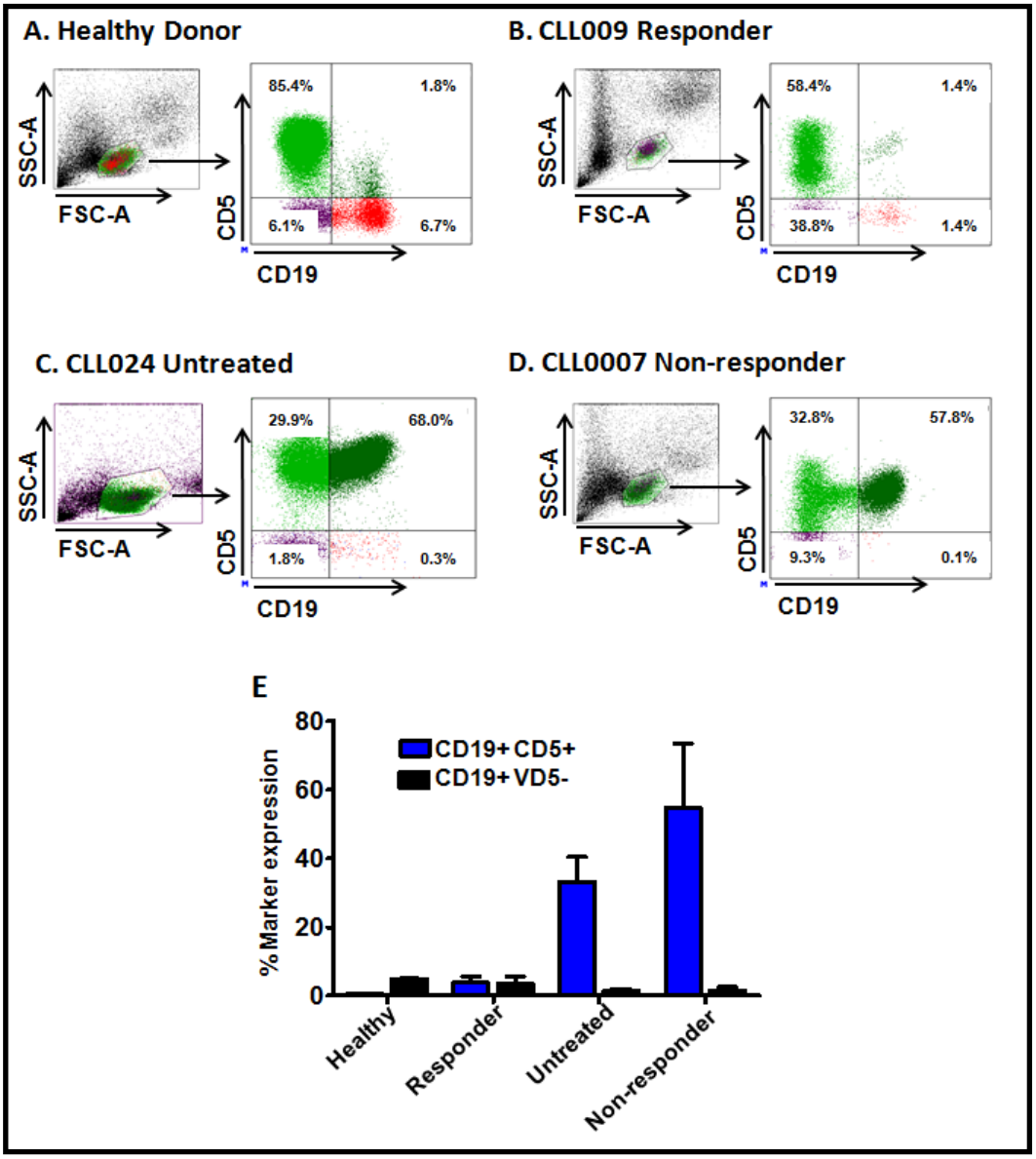

Figure 1 Legend: Flow cytometry analysis of percentages and CD5+ B cells and CD5- B cells, gated on lymphocytes from peripheral blood in **a)** a healthy donor and **b)** responder**, c)** untreated and **d)** refractory (non-responder) and CLL patients. **(e)** Percent marker expression for all patients. Blue bars indicate CD19+ CD5+cells; black bars indicate CD19+ CD5- cells (mean ± SD).

Combination treatment selectively kills CLL cells ex-vivo and improves survival of CLL xenograft model. The leukemic CD19+ CD5+ subpopulation of peripheral blood mononuclear cells (PBMCs) from untreated **(Fig. 2a)** and non-responder/refractory **(Fig. 2b)** CLL patients were examined for cell viability following treatment with a low, sub-optimal dose of ABT-737 (5nM) or by VSV (10 multiplicity of infection - MOI) infection. Treatment with VSV + ABT-737 decreased cell viability to 25% (untreated) and 33.0% (non-responder) by 48h while only minimally reducing the viability of non-leukemic, CD19+ CD5- cells from the same patient to 83% (untreated) and 89% (non-responder), demonstrating the capacity of the combination treatment to specifically kill leukemic cells. Next, we sought to investigate the antitumor efficacy of VSV + ABT-737 combination therapy in a murine A20 lymphoma model, which is partially resistant to VSV treatment (Samuel *et al.*, 2010). Tumors grew aggressively in untreated mice with an average tumor volume of >2000 mm^3 measured 10 days post treatment initiation **(Fig. 2c).** Single agent treatments, ABT-737 or VSV, decreased tumor volume by 32% and 43% respectively. A statistically significant reduction in tumor size ($P \leq 0.05$) was observed for the combination-treated animals compared to single agents or the vehicle. The effect of VSV + ABT-737 on tumor growth translated to increased animal survival (**Fig. 2d)** and a statistically significant increase in median survival time from 10 days (VSV– or ABT-737–treated groups) to 24 days (VSV + ABT-737–treated mice) was observed ($P \leq 0.05$).

Figure 2: ABT-737 + VSV therapy selectively reduces viability of CLL cells ex-vivo and in vivo.

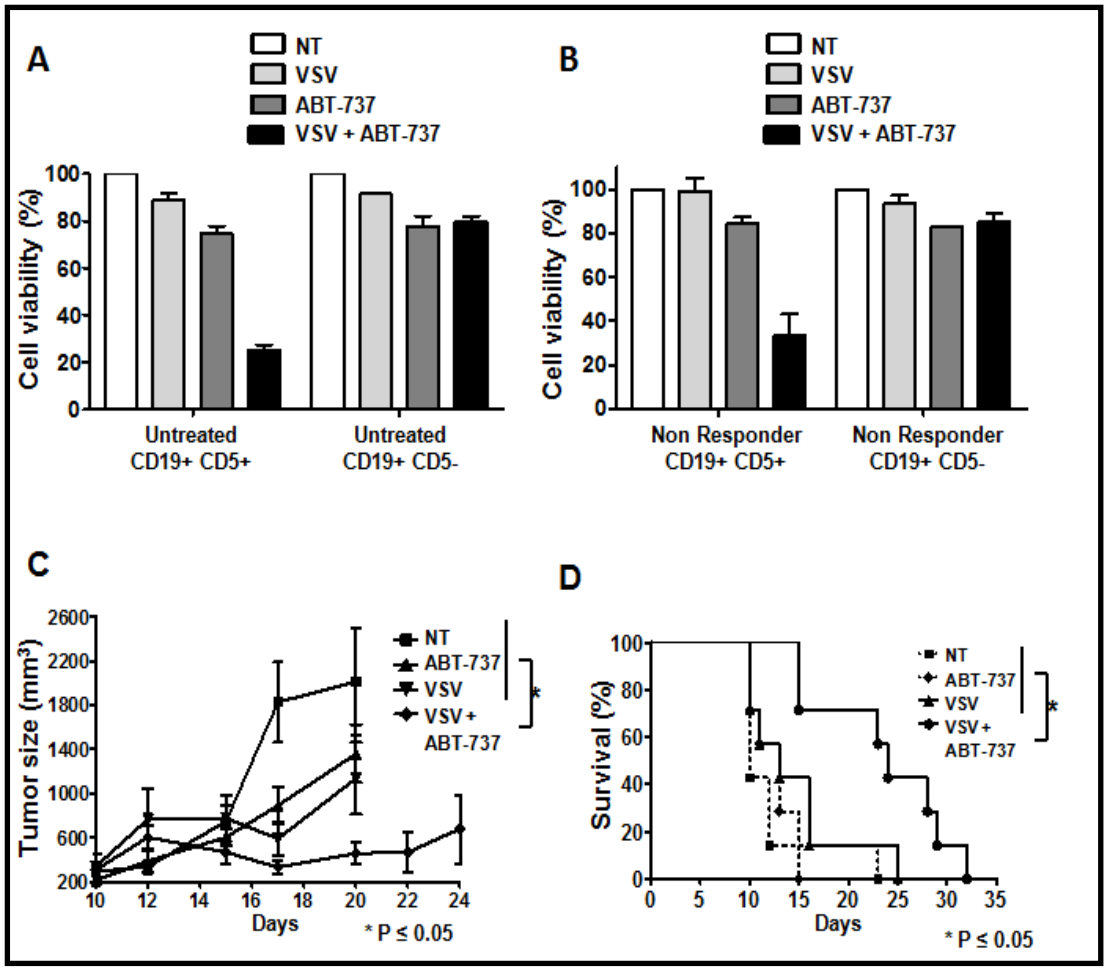

Figure 2 Legend: MTT assay showing sensitivity of CD19+ CD5+ cells of **a)** untreated (n=3) and **b)** non-responder (n=3) CLL patients to VSV + ABT-737 combination treatment in comparison to CD19+ CD5- cells (mean ± SD). White bars indicate control cells; light grey bars indicate VSV infection; dark grey bars indicate ABT-737 treatment and black bars indicate VSV + ABT-737 treatment; NT – non-treated. **(c)** Tumor volumes were calculated as ½(length × width2) and values are expressed as the mean ± SD of tumor volume (n = 7). **(d)** Animals were evaluated for signs of stress such as infection, dehydration, weight loss, and limb paralysis. Mice were sacrificed when tumor volumes exceed 2000 mm3. Stars (*) indicate $P < 0.05$ comparing tumor size between the single and combination treatment groups.

Gene expression profiles in CLL cells. We performed analysis of transcriptional profiles of leukemic CD19+ CD5+ population exposed to Obatoclax, VSV or the combination and compared them to untreated CLL cells as a tool to investigate the biological responses of CLL cells to either single agents or the combination. Comparisons of expression profiles between VSV vs. control (vehicle-treated) cells, Obatoclax vs. control cells, and VSV + Obatoclax vs. control cells were generated and differentially regulated pathways were evaluated using Ingenuity systems pathway analysis (IPA) **(Fig. 3a).** Expression profile comparisons revealed changes in several pathways, including changes in mTOR and apoptotic signaling pathways, which were the only differentially regulated pathways common for the three comparisons. Expression profile analysis of individual genes **(Fig. 3b)** of the apoptotic pathway revealed decreases in anti-apoptotic BIRC6 (Obatoclax- and combination-treated cells), and increases in both pro-apoptotic BID and the inhibitor of apoptosis (IAP)-binding protein, DIABLO (VSV- and combination-treated cells). Changes in transcription of genes belonging to mTOR pathway **(Fig. 3c)** revealed down-regulation in mRNA levels of PRKAB2 only in CLL cells exposed to Obatoclax + VSV combination. The protein encoded by this gene is a regulatory subunit of the AMP-activated protein kinase, an enzyme that monitors cellular energy status (Zaha *et al.*, 2012).

To further evaluate differentially regulated genes and pathways, we analyzed differences in expression profiles of genes belonging to other top ten statistically significant pathways based on IPA that are unique to each treatment. Exposure of cells to VSV alone **(Fig. 3a)** induced up-regulation of numerous genes that belong to oxidative phosphorylation (45/159 or 28.3 %) and mitochondrial dysfunction (38/174 or 21.8 %) including ATPases, ATP synthases, NADH dehydrogenases, and cytochrome c oxidases. Network analysis of cytochrome c oxidases and cyclooxygenases (both in functional relationship with BCL-2) revealed that in addition to up-regulation of numerous electron transport chain proteins, the expression of several anti-oxidant peroxiredoxin enzymes is also increased which may indicate induction of reactive oxygen species upon VSV exposure**.** Interestingly,

the data above indicate that the most prominent effects of VSV infection in CLL cells are changes in mitochondrial function and oxidative phosphorylation. In cells exposed to Obatoclax **(Fig. 3a)** we observed changes in mRNA levels of several proteins that are involved in regulation of transcription (Estrogen receptor signaling), mostly down-regulation of complex subunit moderators and RNA polymerases. Moreover, a subset of down-regulated molecules clustered to B cell receptor signaling pathway, belong to a pro-survival mitogen-activated protein kinase (MAPK) pathway. Together, these data indicate that the treatment of CLL cells with Obatoclax induces down-regulation of pro-survival signaling. Transcriptional profiling in cells exposed to VSV + Obatoclax revealed differences in the expression of a subset of genes that belong to receptor signaling and metabolic regulation, revealing down-regulation of several MAPKs, inositol metabolizing proteins and several acyl-CoA dehydrogenase family members. Overall, these data indicate that in CD19+ CD5+ cells, changes in expression of genes that are involved in mTOR and apoptotic pathways are observed in all three treatment groups (VSV, Obatoclax or the combination) and are likely the most prominent biological response to therapies, although pathways that regulate metabolism, protein synthesis and pro-survival signaling also contribute to such responses. We did not observe dramatic changes in IFN antiviral response genes, which is likely due to defective IFN signaling frequently observed in cancer cells and specifically in primary CLL (Stojdl *et al.*, 2000; Tomic *et al.*, 2011; Wong *et al.*, 1997).

Figure 3: Analysis of gene expression profiles in CLL cells.

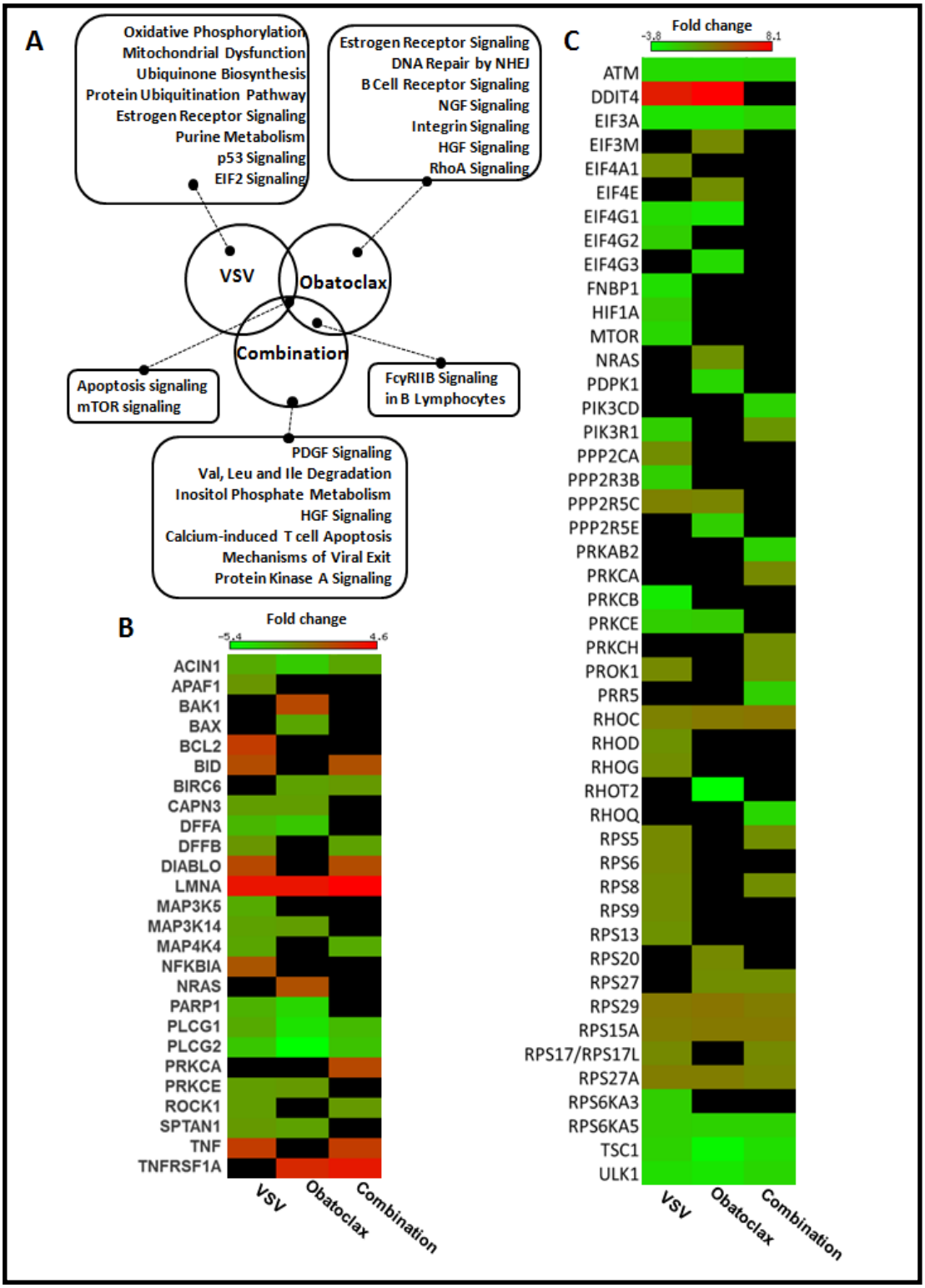

Figure 3 Legend. (a) For each cluster, the differentially regulated pathways are indicated, and were compared to non-treated cells. Genes that belong to multiple Clusters of Orthologous Groups (COG) were placed into each assigned COG category. **b)** and **c)** The heatmaps show statistically significant differentially regulated genes for apoptosis **(b)** and mTOR **(c)** pathways. The genes shown in the heatmaps are derived from the following comparisons (left to right): VSV to control, Obatoclax to control, and VSV + Obatoclax to control and were selected based on the following criteria: absolute fold change >1.3 and nominal p-value <0.05. Missing values that did not pass these criteria are shown in black.

Combination treatment activates markers of autophagy. Next we examined the markers of autophagy in CLL cells exposed to Bcl-2 inhibitor and VSV. The combined therapeutic effect observed in cells treated with VSV + ABT-737 (75% cytotoxicity) was partially reversed by the late stage autophagy-lysosomal inhibitor chloroquine (CQ) **(Fig 4a)**, without a significant change in viral replication **(Fig 4b)**, suggesting a role of autophagy in CLL cell death induced with VSV + ABT-737. We next evaluated several markers of autophagy, including protein levels of mTOR and phosphorylation of FOXO3A in CLL cells exposed to either single or combination therapies. Two hallmarks of autophagy were also measured - conversion of LC3-I (19 kDa) to LC3-II (17 kDa) and p62 degradation **(Fig. 4c)**. At 24 h post infection, the levels of LC3-II increased 5-fold in VSV + Obatoclax treated cells when compared to non-treated cells and p62 decreased to undetectable levels, indicating the formation of autophagosomes and an increase in protein flux, respectively. Autophagy is regulated by a variety of cell signaling pathways including mTOR (Kim *et al.*, 2011). Functionally, activated mTOR suppresses the initiation of the autophagic pathway. A 3-fold decrease in mTOR protein levels was observed in VSV + Obatoclax treated cells **(Fig. 4c).** FOXO3A, a transcriptional regulator implemented in tumorigenesis, stimulates autophagy when activated by dephosphorylation (Chiacchiera *et al.*, 2009, 2010). FOXO3A phosphorylation at serine residue 253 was reduced by 3-fold in the presence of VSV + Obatoclax **(Fig. 4c).** Similar to Obatoclax + VSV combination treatment, LC3-I was proteolytically cleaved yielding LC3-II (2-fold increase) and p62 was completely degraded in cells exposed to ABT-737 + VSV **(Fig. 4d)**. mTOR levels were reduced by 3-fold and serine 253 dephosphorylation of FOXO3A diminished by 2-fold with VSV + ABT-737 treatment **(Fig. 4d).** Collectively, these results demonstrate the induction of the autophagy in CLL cells treated with VSV+ BCL-2 inhibitor.

It has previously been reported that overexpression of pro-survival BCL-2 family members inhibits the induction of apoptosis and autophagy in cancer cells (Sinha *et al.*, 2008). Immunoblot analysis showed that BCL-2 was overexpressed in PBMCs isolated from a CLL patient, compared to a healthy volunteer; BCL-2 and MCL-1

were also overexpressed in human B-cell non-Hodgkin's lymphoma Karpas-422 cell line **(Fig. 5a**). Karpas-422 cells display similar characteristics to primary CLL cells and are refractory to VSV oncolysis (Samuel *et al.*, 2010; Tumilasci *et al.*, 2008). It has been suggested that disruption of protein-protein interactions between anti-apoptotic BCL-2 and pro-autophagic Beclin-1 could promote autophagic cell death (Pattingre *et al.*, 2006; Pattingre *et al.*, 2005; Sinha *et al.*, 2008). Co-immunoprecipitation experiments revealed that BCL-2/Beclin-1 and MCL-1/Beclin-1 heterodimer complexes were constitutively present in untreated Karpas-422 cells **(Fig. 5b, lane 1)**. VSV or Obatoclax alone had minimal effect on heterodimer formation **(Fig. 5b, lane 2, 3),** but VSV + Obatoclax almost completely abrogates BCL-2 and MCL-1 interactions with Beclin-1 **(Fig. 5b, lane 4)**. Collectively, these results suggest Obatoclax disrupts BCL-2- and MCL-1/Beclin-1 interactions, but only in the presence of VSV. Next, we immunoprecipitated BCL-2 and measured Beclin-1 displacement in cells treated with ABT-737 + VSV. Such treatment neutralized BCL-2/Beclin-1 inhibitory interactions allowing the release of Beclin-1 **(Fig. 5c, lane 4)**. These results suggest that ABT-737 in combination with VSV disrupt BCL-2/Beclin-1 interactions which likely facilitates induction of both apoptosis and autophagy. Based on the data above, both inhibitors, Obatoclax and ABT-737, utilize a comparable mechanism to potentiate VSV-mediated oncolysis in CLL cells

Figure 4: BCL-2 inhibitor + VSV combination induces markers of autophagy in CLL cells.

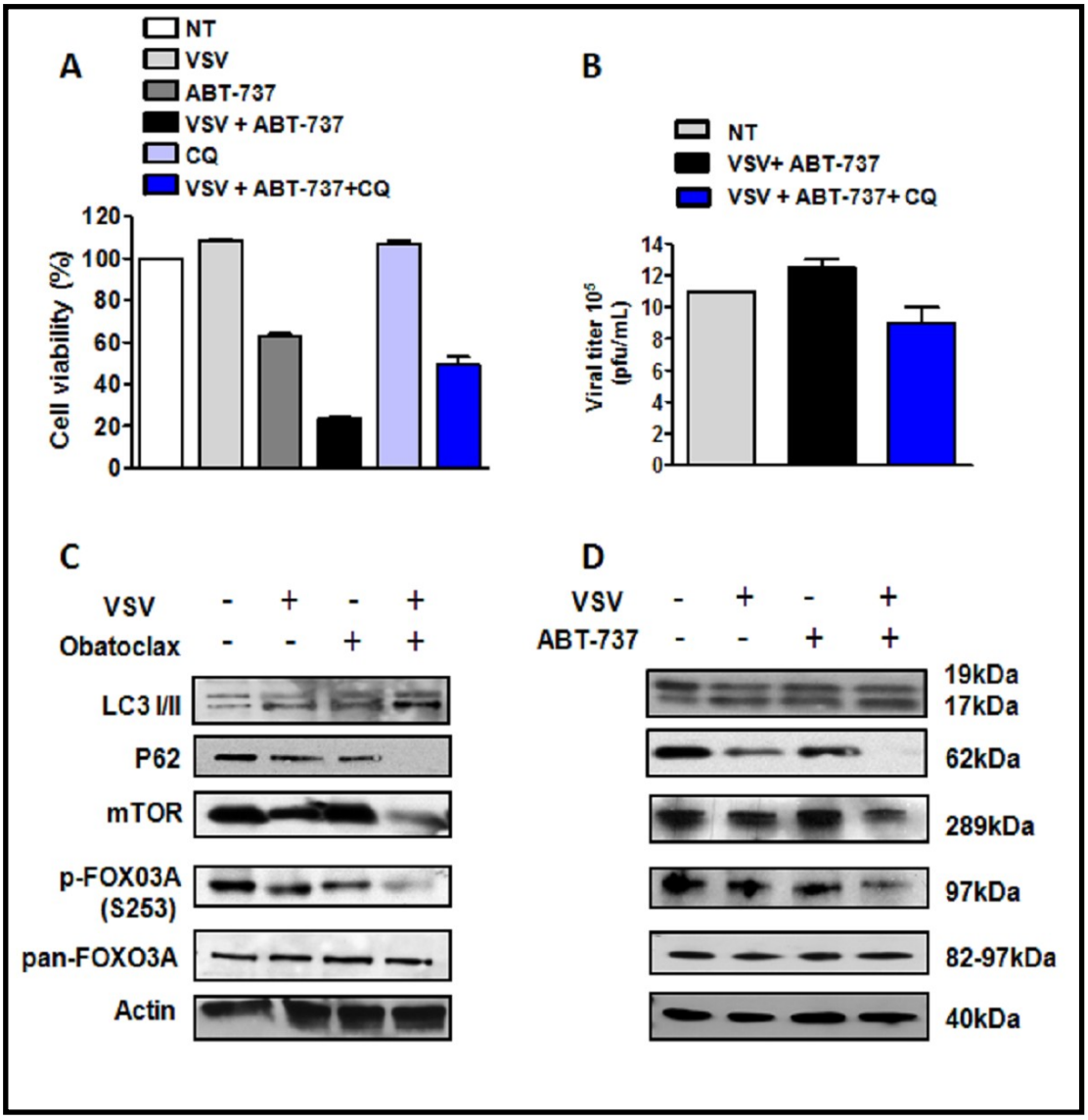

Figure 4 Legend: (a) MTT assays showing sensitivity of CLL cells of a CLL patient to VSV (10 MOI) and ABT-737 (5nM) combination treatment with or without 30 min pre-treatment with autophagy inhibitor chloroquine (CQ). White bars indicate control cells; light grey bars indicate VSV infection; dark grey bars indicate ABT-737 treatment, black bars indicate VSV + ABT-737 treatment, light blue bars indicate CQ treatment and dark blue bars indicate VSV + ABT-737 + CQ treatment. **(b)** Viral titer was examined by plaque assay (mean ± SD). Light grey bars indicate VSV infection; black bars indicate VSV + ABT-737 treatment, and dark blue bars indicate VSV + ABT-737 + CQ treatment; pfu – plaque forming units **(c, d)** 24h post-treatment, CLL cells were lysed and LC3-II accrual and p62 degradation were analyzed by immunobloting. LC3-I (19 kDa), LC3-II (17 kDa), p62 (62 kDa), mTOR (289 kDa), phospho-FOXO3A (S243) (97 kDa), total FOXO3A (82-97 kDA) and actin (40 kDa).

Figure 5: Disruption of Beclin-1/BCL-2 and Beclin-1/MCL-1 interactions

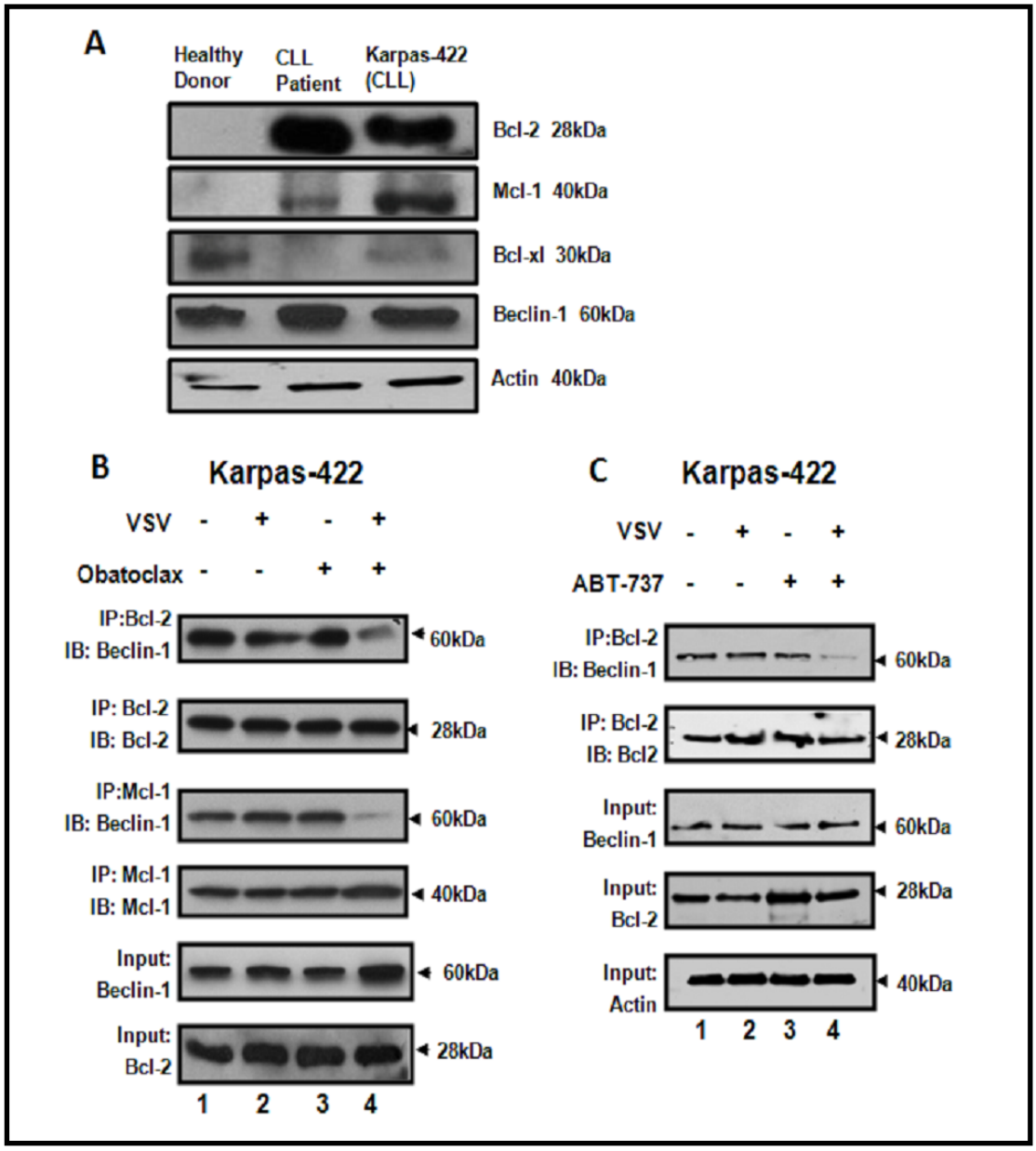

Figure 5: Legend. (a) Anti-apoptotic BCL-2, MCL-1 and BCL-XL as well as Beclin 1 levels were analyzed by immunobloting in PBMCs isolated from a healthy donor, a CLL patient, and in Karpas-422 cell line. Karpas-422 cells were treated with Obatoclax and VSV **(b)** or ABT-737 and VSV **(c)** for 24h. Cells were lysed in 1% CHAPS lysis buffer and BCL-2 and MCL-1 were immunoprecipitated followed by immunoblotting for Beclin-1.

Combination treatment induces Atg5-mediated cell death and autophagosome formation. To confirm the role of autophagy in VSV + ABT-737-mediated cell killing, mouse embryonic fibroblasts (MEFs) derived from wild type (WT) and autophagy-deficient Atg5 knockout (KO) mice were treated with VSV and/or ABT-737 and cytotoxicity was measured by MTT assay. Both WT and KO cell lines displayed complete resistance to ABT-737 (5nM) treatment after 24h **(Fig. 6a)**. VSV-mediated lysis is markedly decreased in Atg5 KO MEFs in comparison to WT cells. Cell viability decreased from 100% to 24% with VSV infection (0.01 MOI) in WT MEFs compared to 67% in Atg5 KO cells 24h post infection. Combining ABT-737 with VSV did not further decrease cell viability in WT MEFs compared to VSV alone **(Fig. 6a)** (22% VSV alone and 21% VSV ± ABT-737**)**; slightly better therapeutic effect for the combination was observed in the KO cell line. Viral titer was examined by plaque assay and a 1 log decrease was observed in Atg5 KO MEFs compared to Atg5 WT MEFs **(Fig. 6b).** Thus, viral replication is augmented in the WT cell line (**Fig 6b,c**) and VSV oncolysis appears to be in part dependent on autophagy. Immunoblot studies revealed that LC3-II did not accumulate in Atg5-/- cells but was evident in Atg5+/+ cells with combination treatment (**Fig 6c, top panel**). These results were validated by immunofluorescence studies using green-fluorescent protein (GFP)-LC3 as a label for autophagosome formation. Combination VSV + ABT-737 induced both GFP-LC3 positive autophagic vesicles and punctate foci in Atg5 WT MEFs **(Fig. 6d).** As expected, vesicle formation and foci were not detected in Atg5 KO MEFs. Altogether these results imply that VSV + ABT-737-induced cell death is in part dependent on autophagy.

Figure 6: VSV + ABT-737 combination treatment induces Atg5-mediated cell death.

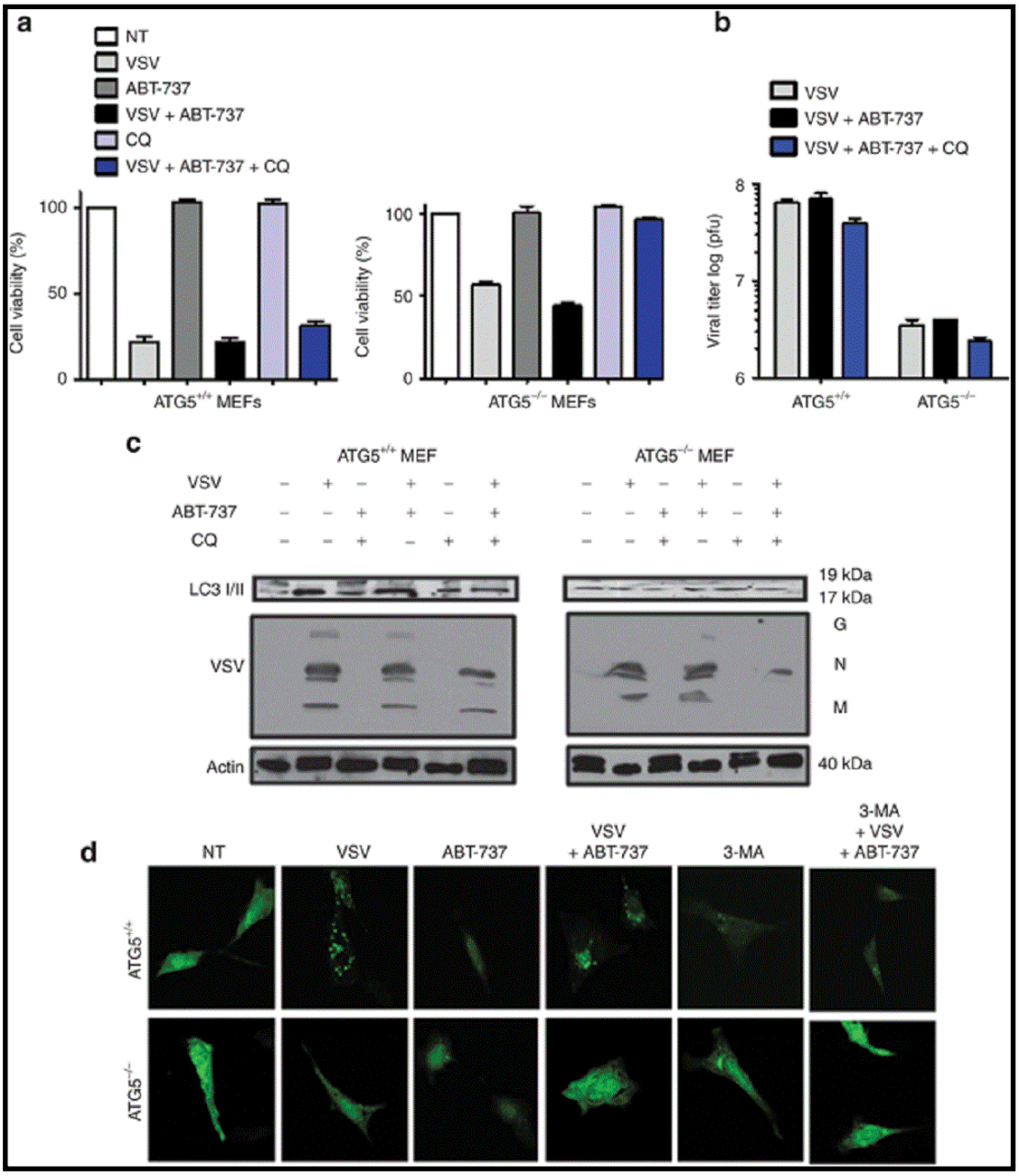

Figure 6 Legend. **(a)** Cell viability analysis was performed on WT MEFs and Atg5 KO MEFs treated for 24h with VSV (0.1 MOI) and ABT-737 (5nM) alone and in combination. Cells were treated or not with CQ (10uM) for 30 min prior to VSV + ABT-737 therapy. White bars indicate non-treated cells; light grey bars indicate VSV infection; dark grey bars indicate ABT-737 treatment; black bars indicate VSV + ABT-737 treatment; light blue bars indicate CQ treatment and dark blue bars indicate VSV + ABT-737 +CQ treatment. **(b)** WT Atg5 KO MEFs were infected with VSV or ABT-737 alone and in combination followed by inhibition with CQ. 24h after infection, the cells and culture supernatants were recovered, and the levels of viral titer were examined by the plaque assay. Viral titers were significantly lower in Atg5 KO MEFs. Light grey bars indicate VSV infection; black bars indicate VSV + ABT-737 treatment, and dark blue bars indicate VSV + ABT-737 + CQ treatment **(c)** Western blot measures processing of LC3 and VSV replication in lysates after 24 h of VSV infection (0.1MOI). LC3-I (19 kDa) and LC3-II (17 kDa). Viron G, glycoprotein; M, matrix; N, nucleocapsid. **(d)** WT (top panel) and KO (bottom panel) Atg5 MEFs were transfected with LC3-GFP plasmid, treated with VSV ± ABT-737 and analysed by immunofluorescence. 3-MA - 3-methyladenine (autophagy inhibitor). Green spots (foci) indicate LC3-GFP localized at autophagosomes.

Upregulation of apoptotic and autophagic markers in Karpas-422 cells exposed to combination treatment. Karpas-422 cells were examined for autophagy and apoptosis induction by Cyto-ID staining (selective label of autophagic vacuoles), p62 expression, and annexin V staining by flow cytometry analysis. Following VSV + ABT-737 combination, Cyto-ID+ cells increased from 13% in non-treated samples to 29% **(Fig. 7a, top panel).** The corresponding p62 levels were reduced significantly from 93% in non-treated cells to 33% in cells treated with the combination **(Fig. 7a, middle panel),** thus confirming stimulation of the autophagic pathway. Combination treatment also dramatically increased annexin V-positive staining from 7% to 62% in Karpas-422 cells (**Fig. 7a, bottom panel).** The Pearson's correlation test revealed a strong association (R^2=0.8698) between Cyto-ID staining and p62 expression **(Fig. 7b)**. We subsequently identified Cyto-ID+ and p62- cells as autophagic. To examine the relationship between the apoptotic and autophagic pathways we measured early and late apoptosis markers, annexin V and cleaved caspase 3, respectively, and mitochondrial membrane potential ($\Delta\Psi$) using 3,3'-dihexyloxacarbocyanine iodide (DIOC6(3)) in autophagic cells (Cyto-ID+ and p62–). **(Fig. 7c);** DIOC6(3) is used to label living cells. As shown in **Fig. 7c,** 5% of autophagic Karpas-422 cells stained positive for annexin V with ABT-737 single agent treatment and 2% with VSV infection, and an increase to 40% was detected in the combination treated cells. Similarly, cleaved caspase 3 staining increased from 5% and 1% to 46% in autophagic Karpas-422 cells. Loss of mitochondrial membrane potential (DIOC6(3) low) increased from 2% in non-treated cells to 44% with VSV + ABT-737 treatment in these cells **(Fig. 7c).** Pre-treatment for 1h with the pan-caspase inhibitor zVAD-FMK (100uM) inhibited apoptosis in autophagic combination-treated cells **(Fig. 7c)** reducing annexin V, cleaved caspase 3, and DIOC6(3) low levels from 40% to 4%, 46% to 1% and 44% to 8% respectively. Moreover, chemical inhibition of the autophagic pathway increased activation of the apoptotic pathway; when 3-methyladenine (3-MA) (10mM) was administered to combination treated autophagic cells death markers increased from 40% to 67% (annexin V), from 46% to 73% (caspase 3) and from 44% to 75% (DIOC6(3) low) in autophagic cells **(Fig. 7c)**.

These findings confirm that both apoptotic and autophagic pathways contribute to CLL cell death.

Figure 7: ABT-737 + VSV induces markers of autophagy.

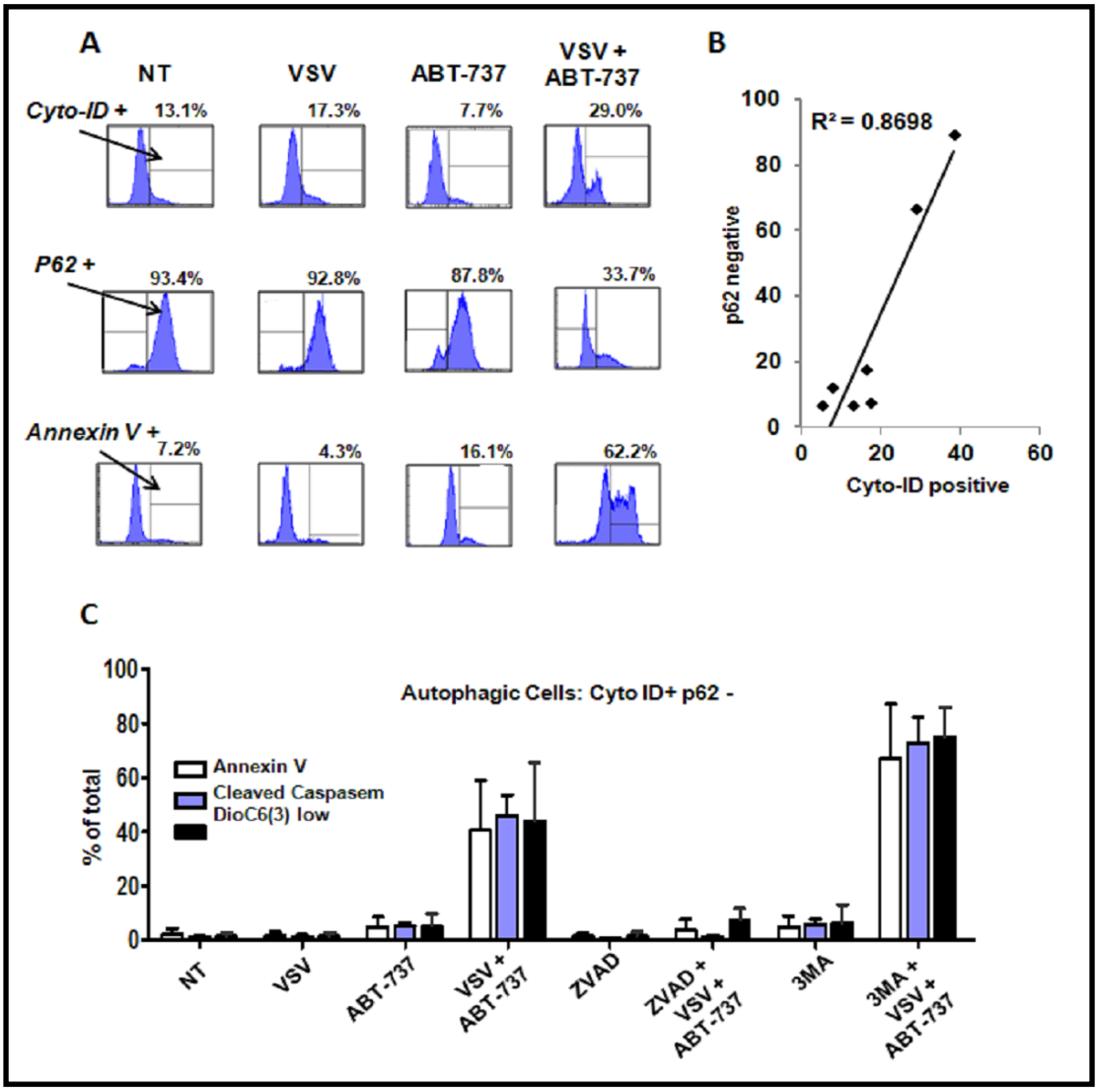

Figure 7 Legend. Karpas-422 cells were infected with VSV (10 MOI) and ABT-737 (150nM) alone or in combination; 18 h after infection, the cells and culture supernatants were recovered and stained with **a)** Cyto-ID, p62, or annexin V antibody. **(b)** Pearson correlation was used to quantify the degree of association between p62 and Cyto-ID. **(c)** Annexin V, cleaved caspase 3 and DIOC6(3) low levels were monitored by flow cytometry. White bars indicate annexin V; blue bars indicate cleaved caspase and black bar indicate DIOC6(3) low staining. zVAD (apoptosis inhibitor); 3-MA=3-methyladenine (autophagy inhibitor); NT - non-treated. All experiments were performed in triplicate.

Discussion

CLL accounts for about one-third of all leukemias, with an estimated 16,060 new cases and 4,580 deaths in USA alone in 2012 and no curative therapies are available (Eichhorst *et al.*, 2011; Hallek, Cheson*, et al.*, 2008; Hallek & German, 2008; Hallek *et al.*, 1997; Shanshal *et al.*, 2012). These statistics underline the unmet need for new alternative strategies to target CLL and improve survival. In the present manuscript, we demonstrate that the combination of VSV and BCL-2 inhibitors: 1) induce cell death in leukemic CLL cells from patients that had not responded to standard of care therapies; 2) induce changes in expression of genes involved in the regulation of mTOR, apoptotic, and metabolic pathways; 3) trigger both autophagy and apoptosis in CLL cells; 4) enhance Atg5-dependent and Beclin 1-mediated cell killing; and 5) degrade mTOR and decrease FOXO3A phosphorylation.

The ABT-737 + VSV combination therapy was also evaluated in a murine model of lymphoma (**Fig. 2a**). Although modest effects were observed with single agent - ABT-737 or VSV - treatments, therapeutic benefit and prolonged survival was observed in combination treated animals without overt toxicity, indicating that such therapeutic combination selectively kills tumor cells *in vivo*. Microarray analysis was performed to gain insight into the mechanism(s) responsible for therapeutic efficacy and selectivity of VSV + BCL-2 inhibitor combination. Changes in transcription profiles and clustering of such changes into pathways revealed differential regulation of mTOR, apoptotic, and metabolic pathways in CLL cells exposed to either single or combined treatments compared to non-treated cells. Differential regulation was also observed in genes involved in protein translation, oxidative stress and pro-survival pathways. These data indicate that CLL cells exposed to combination therapies likely undergo changes in protein translation, cellular metabolism and survival/apoptosis pathway signaling. Activation of IFN-responsive antiviral pathways were not observed in our array analysis; such pathways are significantly diminished or aberrant in many cancers including in CLL (Tomic *et al.*, 2011) which

could explain the lack of antiviral genes observed in the microarray following VSV infection. Interestingly, only a handful of genes in CD19+ CD5- (non-leukemic) cell population exposed to VSV, Obatoclax or the combination had altered gene expression, indicating that healthy cells do not activate similar stress-response mechanisms in response to such therapies (data not shown).

BCL-2 has a dual function to regulate both apoptosis and autophagy [10,17-19]. Anti-apoptotic BCL-2 members have been shown to inhibit Beclin 1 induced autophagy. Executioner caspases can cleave Beclin 1 and Atg5 inactivating the autophagic pathway (Kang *et al.*, 2011). Conversely Beclin-1 can inhibit activation of caspases and BID protein. Consistent with these conclusions, we observed a strong association between apoptosis and autophagy: inhibition of apoptosis with zVAD completely abrogated VSV + ABT-737 induced apoptosis in autophagic (Cyto ID+, p62-) Karpas-422 cells **(Fig 7c)**, and inhibition of autophagy with 3-MA enhanced the apoptotic effect of the combination-induced apoptosis. Unfortunately, little is known about the role of autophagy in VSV-induced lysis of cancer cells. Here we demonstrate a major role of autophagy in VSV-mediated CLL cell death. Because of its dual role in inhibiting both apoptosis and autophagy, BCL-2 represents an attractive target for development of therapies that will stimulate both pathways.

We propose a mechanism in which CLL cells exposed to VSV undergo metabolic and oxidative stress that leads to changes in protein translation and cellular metabolism. Such changes alone do not activate autophagy, probably because of the tight association between Beclin-1 and BCL-2. However, upon addition of BCL-2 inhibitors, VSV-induced cellular stress initiates the autophagic pathway, in part because Beclin-1 is available to participate in the scaffolding and initiation of autophagosome formation. These data are further corroborated by co-immunoprecipitation studies demonstrating that inhibition of the autophagic pathway in CLL is due to overexpression of pro-survival BCL-2 family members. Disruption of the BCL-2/Beclin 1 complex indicates that autophagy is inhibited in CLL cells and that activation of the autophagic pathway by combination treatment promotes

tumor killing. In conclusion, we show for the first time that oncolytic VSV induces cell death in CLL cells via activation of both autophagic and apoptotic signaling and demonstrate a mechanism of cell death that is in part dependent on autophagy. Due to the dose limiting cytotoxity of BCL-2 agonists as single agents, low dose combination with VSV provides an alternative strategy that may have a greater safety profile for the treatment of CLL and other malignancies that could be applied in clinics.

Material and Methods

Patients and PBMC isolation. PBMCs were obtained from healthy individuals and CLL patients at the Robert & Carol Weissman Cancer Center at Martin Memorial Health Systems (Stuart, FL, USA) the Vaccine & Gene Therapy Institute of Florida (Port St. Lucie, FL, USA) and the Jewish General Hospital (Montreal, QC, Canada) following written informed consent, in agreement with the Vaccine & Gene Therapy Institute of Florida, Jewish General Hospital and McGill University Research Ethics Committee. The absolute lymphocyte counts were typical of CLL patients in general. PBMCs were isolated as previously described (Samuel *et al.*, 2010; Tumilasci *et al.*, 2008). PBMCs were cultured in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (Wisent, St-Bruno, Quebec, Canada) and 100 U/ml penicillin–streptomycin. PBMCs were cultured at 37 °C in a humidified, 5% CO2 incubator.

Cell lines. The human B-lymphoma cell line Karpas-422 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the A20 mouse B-lymphoma cell line was purchased from ATCC (Manassas, VA). Atg5 wild-type (WT) (Atg5 +/+.) and Atg5 knockout (KO) (Atg5 -/-) mouse embryonic fibroblasts (MEFs) were obtained from Dr. Nathalie Grandvaux, CRCHUM-Centre Hospitalier de l'Université de Montréal , Montréal, CA, with the permission of Dr. Noboru Mizushima, Tokyo Medical and Dental University, Tokyo, JP., who generated the original Atg5 WT and KO MEFs. For all experiments early passage primary MEFs were used. Karpas-422 and A20 cell lines were grown in RPMI 1640 medium (Wisent, St Bruno, QC, Canada) supplemented with 10% fetal calf serum, penicillin and streptomycin. Atg5 WT and KO MEFS were cultured in DMEM medium (Wisent, St Bruno, QC, Canada) supplemented with 10% fetal calf serum, penicillin and streptomycin. All cell lines were maintained at 37 °C and 5% CO_2.

Virus production, quantification and infection. Construction of VSV was previously described(Stojdl *et al.*, 2003). Virus stock was grown in Vero cells

(purchased from ATCC, Bethesda, MD), concentrated from cell-free supernatants by centrifugation (15,000 r.p.m./4 °C/90 minutes) and titrated in duplicate by standard plaque assay as previously described(Tumilasci *et al.*, 2008). Primary PBMC isolates and Karpas-422 cells were infected with VSV at a multiplicity of infection of 10 plaque-forming units/cell for 1 hour in serum-free media at 37 °C. The cells were then incubated with complete medium at 37 °C for the indicated times.

Viability assay. Cell viability was assessed by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) dye absorbance according to the manufacturer's instructions (Chemicon, Billerica, MA) as previously described(Samuel *et al.*, 2010) .For drug combination studies, cells were incubated with or without Obatoclax (100 nmol/l) or ABT-737 (150nmol/l, Karpas-422, 5nmol/l PBMCs) and infected or not with VSV (10 multiplicity of infection) as indicated. Plates were incubated at 37 °C, 5% CO_2 and cells analyzed every 24 hours for 3 days. Each experimental condition was performed in triplicate. Cell viability was also analyzed by annexin V, cleaved caspase 3 and 3,3'-Dihexyloxacarbocyanine Iodide (DIOC6(3)) staining by flow cytometry analysis.

Protein extraction and western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline, and proteins were extracted as described previously (Samuel *et al.*, 2010; Tumilasci *et al.*, 2008).Membranes were blocked for 1 hour in 5% nonfat dried milk in TBST (Tris-buffered saline + 0.5% Tween-20). Followed by incubation with any of the following primary antibodies: mTOR, FOXO3A-S256 , and Beclin-1 (Cell Signaling Technologies, Danvers, MA; 1:2,000); ß-actin (Millipore); LC3 (Novus Biologicals; Oakville, ON), p62 (Santa Cruz Biotechnology; CA; 1:2000) BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2,000), BAX (Santa Cruz Biotechnology; CA;1 µg/ml) and NOXA (Calbiochem, San Diego, CA; 1 µg/ml). For LC3 protein extraction cell pellets were lysed in ice-cold buffer containing 20 mmol/l Tris, 400 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton-X, 30 mmol/l NaF, 40 mmol/l ß-glycerophosphate, 10 mmol/l Na3VO4, 0.1mmol/l PMSF, 1mmol/l DTT and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) in 1/1,000 dilution. Extracts were vortexed for 30min-1hr at 4 °C and centrifuged at 10,000 g for 10 minutes (4 °C); supernatants were stored at -80 °C.

Protein concentration was determined with Bio-Rad protein assay reagent (BioRad, Hercules, CA). Protein extracts were resolved using mini-PROTEAN TGX precast gels (BioRad, Hercules, CA) and transferred to polyvinylidene difluoride membrane (BioRad, Hercules, CA).

In vivo murine lymphoma model. This study was approved by the local animal care and institutional animal ethics committee of Jewish General Hospital and McGill University. A total of seven animals per treatment group were used for this study, 4–6-week-old female nu/nu mice (Charles River Laboratories, Pointe Claire, QC, Canada) were injected subcutaneously with 1×10^6 A20 cells in a 100-µl volume into the hind flanks. Tumor volumes were measured and calculated as ½(length × width2)1. Once tumors were palpable, animals were randomly assigned to treatment groups and received ten intraperitoneal injections of ABT-737 (75 mg/day/kg). ABT-737 was dissolved in 30% propylene glycol, 5% Tween 80,65% D5W (5% dextrose in water), pH 4-5. At days 2 and 5 following ABT-737 injection, VSV was inoculated intratumorally at 5×10^7 plaque-forming units of virus each. Animals were evaluated for signs of stress such as infection, dehydration, weight loss (>20%), and limb paralysis.

Co-immunoprecipitation of BCL-2 family proteins. Co-immunoprecipitation studies were performed as previously described (Samuel *et al.*, 2010). Membranes were blocked for 1 hour in 5% non-fat dried milk in TBST (Tris-buffered saline + 0.5% Tween-20) followed by incubation with the following primary antibodies: BCL-2, MCL-1 (Santa Cruz Biotechnology; 1:2,000), and Beclin-1 (Cell Signaling Technologies, Danvers, MA; 1:2000)

Autophagy analysis by flow cytometry. Autophagy was also analyzed by Cyto-ID and p62 staining by flow cytomtery. Cyto-ID was used according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). p62 (Santa Cruz Biotechnology; CA) was labeled with Alexa Fluor® 647 using the Zenon® Alexa Fluor® 647 Mouse IgG1 Labeling Kit (Invitrogen, Carlsbad, CA).

Immunofluorescence. Atg5 WT and KO MEFs grown on glass coverslips were transfected with constructs encoding GFP and LC3-GFP using JETPRIME (Polyplus-transfection, Illkirch, FR) according to the manufacturer's instructions.

Following transfection cells were treated or not with ABT-737 and infected with VSV. 6 hours post-infection, coverslips were washed two times in PBS and fixed in PBS containing 4% formaldehyde for 12 min. Coverslips were then washed three times in PBS and mounted on glass slides with Shandon Immuno-Mount (Thermo Scientific, Ottawa, ON), and left to dry overnight at room temperature in the dark.

Statistical analysis. Graphics and statistical analysis were executed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Differences among the treatment groups were analyzed by paired t-test. The P values <0.05 were considered statistically significant. Average values were expressed as mean ± SD.

Microarray analyses. CLL cells from both non-responders and non-treated patients were treated with VSV, Obatoclax or combination and total RNA was isolated from samples (n = 3 per treatment group) using RNeasy Micro Kits (Qiagen). The quantity and quality of the RNA was confirmed using a NanoDrop 2000c (Thermo Fisher) and an Experion Electrophoresis System. Samples (50 ng) were then amplified using Illumina TotalPrep RNA amplification kits (Ambion). The microarray analysis was conducted using 750 ng of biotinylated complementary RNA hybridized to HumanHT-12_V4 BeadChips (Illumina) at 58 °C for 20 h. The arrays were scanned using Illumina's iSCAN and quantified using Genome Studio (Illumina). The analysis of the GenomeStudio output data was conducted using the R and Bioconductor software packages. Quantile normalization was applied, followed by a log2 transformation. The LIMMA package was used to fit a linear model to each probe and perform (moderated) t tests or F tests on the groups being compared. To control the expected proportions of false positives, the FDR for each unadjusted P value was calculated using the Benjamini and Hochberg method implemented in LIMMA. Multidimensional scaling was used as a dimensionality reduction method in R to generate plots for the evaluation of similarities or dissimilarities between datasets. Ingenuity Pathway Analysis software (IPA, Ingenuity Systems) was used to annotate genes and rank canonical pathways. Canonical pathways analysis identified the pathways from the IPA library of pathways that were most significant to the data set. Molecules from the data set that met the cutoff of 1.3 and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the

analysis. The significance of the association between the data set and the canonical pathway was measured by determining of a ratio between the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

ACKNOWLDGEMENT: We thank GeminX for the generous gift of Obatoclax. We also thank AbbVie Laboratories for providing the ABT-737 compound. This work was supported by Canadian Oncolytic Virus Consortium.

CONFLICT OF INTEREST: There is no conflict of interest to disclose

References

1. Shanshal M, Haddad RY. Chronic lymphocytic leukemia. *Dis Mon* 2012; **58**(4)**:** 153-67.

2. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *The New England journal of medicine* 2005; **352**(8)**:** 804-15.

3. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H *et al.* Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 2008; **111**(12)**:** 5446-56.

4. Buggins AG, Pepper CJ. The role of Bcl-2 family proteins in chronic lymphocytic leukaemia. *Leuk Res* 2010; **34**(7)**:** 837-42.

5. Garcia-Saez AJ. The secrets of the Bcl-2 family. *Cell death and differentiation* 2012; **19**(11)**:** 1733-40.

6. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. *Journal of cell science* 2009; **122**(Pt 4)**:** 437-41.

7. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Molecular cell* 2010; **37**(3)**:** 299-310.

8. Levine B, Sinha S, Kroemer G. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy* 2008; **4**(5)**:** 600-6.

9. Pattingre S, Levine B. Bcl-2 inhibition of autophagy: a new route to cancer? *Cancer research* 2006; **66**(6)**:** 2885-8.

10. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N *et al.* Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005; **122**(6)**:** 927-39.

11. Sinha S, Levine B. The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene* 2008; **27 Suppl 1:** S137-48.

12. Gordy C, He YW. The crosstalk between autophagy and apoptosis: where does this lead? *Protein & cell* 2012; **3**(1)**:** 17-27.

13. Hou W, Han J, Lu C, Goldstein LA, Rabinowich H. Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. *Autophagy* 2010; **6**(7)**:** 891-900.

14. Zhou F, Yang Y, Xing D. Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *The FEBS journal* 2011; **278**(3)**:** 403-13.

15. Townsend KN, Hughson LR, Schlie K, Poon VI, Westerback A, Lum JJ. Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. *Immunological reviews* 2012; **249**(1)**:** 176-94.

16. Eager RM, Nemunaitis J. Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther* 2011; **18**(5)**:** 305-17.

17. Rowan K. Oncolytic viruses move forward in clinical trials. *Journal of the National Cancer Institute* 2010; **102**(9)**:** 590-5.

18. Breitbach CJ, Burke J, Jonker D, Stephenson J, Haas AR, Chow LQ *et al.* Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 2011; **477**(7362)**:** 99-102.

19. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nature biotechnology* 2012; **30**(7)**:** 658-70.

20. Donnelly OG, Errington-Mais F, Prestwich R, Harrington K, Pandha H, Vile R *et al.* Recent clinical experience with oncolytic viruses. *Current pharmaceutical biotechnology* 2012; **13**(9)**:** 1834-41.

21. Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S *et al.* VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer cell* 2003; **4**(4)**:** 263-75.

22. Lichty BD, Stojdl DF, Taylor RA, Miller L, Frenkel I, Atkins H *et al.* Vesicular stomatitis virus: a potential therapeutic virus for the treatment of hematologic malignancy. *Human gene therapy* 2004; **15**(9)**:** 821-31.

23. Barber GN. Vesicular stomatitis virus as an oncolytic vector. *Viral immunology* 2004; **17**(4)**:** 516-27.

24. Lichty BD, Power AT, Stojdl DF, Bell JC. Vesicular stomatitis virus: re-inventing the bullet. *Trends in molecular medicine* 2004; **10**(5)**:** 210-6.

25. Samuel S, Tumilasci VF, Oliere S, Nguyen TL, Shamy A, Bell J *et al.* VSV oncolysis in combination with the BCL-2 inhibitor obatoclax overcomes apoptosis resistance in chronic lymphocytic leukemia. *Mol Ther* 2010; **18**(12)**:** 2094-103.

26. Tumilasci VF, Oliere S, Nguyen TL, Shamy A, Bell J, Hiscott J. Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary chronic lymphocytic leukemia cells to vesicular stomatitis virus-induced oncolysis. *Journal of virology* 2008; **82**(17)**:** 8487-99.

27. Gaddy DF, Lyles DS. Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx. *Journal of virology* 2007; **81**(6)**:** 2792-804.

28. Mason KD, Khaw SL, Rayeroux KC, Chew E, Lee EF, Fairlie WD *et al.* The BH3 mimetic compound, ABT-737, synergizes with a range of cytotoxic chemotherapy agents in chronic lymphocytic leukemia. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 2009; **23**(11)**:** 2034-41.

29. High LM, Szymanska B, Wilczynska-Kalak U, Barber N, O'Brien R, Khaw SL *et al.* The Bcl-2 homology domain 3 mimetic ABT-737 targets the apoptotic machinery in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interactions with established drugs. *Molecular pharmacology* 2010; **77**(3)**:** 483-94.

30. Campas C, Cosialls AM, Barragan M, Iglesias-Serret D, Santidrian AF, Coll-Mulet L *et al.* Bcl-2 inhibitors induce apoptosis in chronic lymphocytic leukemia cells. *Experimental hematology* 2006; **34**(12)**:** 1663-9.

31. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005; **435**(7042)**:** 677-81.

32. Bajwa N, Liao C, Nikolovska-Coleska Z. Inhibitors of the anti-apoptotic Bcl-2 proteins: a patent review. *Expert Opin Ther Pat* 2012; **22**(1)**:** 37-55.

33. Nguyen M, Marcellus RC, Roulston A, Watson M, Serfass L, Murthy Madiraju SR *et al.* Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**(49)**:** 19512-7.

34. Pandey R, Chander R, Sainis KB. Prodigiosins as anti cancer agents: living upto their name. *Current pharmaceutical design* 2009; **15**(7)**:** 732-41.

35. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S *et al.* ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer research* 2008; **68**(9)**:** 3421-8.

36. Gandhi L, Camidge DR, Ribeiro de Oliveira M, Bonomi P, Gandara D, Khaira D *et al.* Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011; **29**(7)**:** 909-16.

37. Paik PK, Rudin CM, Brown A, Rizvi NA, Takebe N, Travis W *et al.* A phase I study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in solid tumor malignancies. *Cancer chemotherapy and pharmacology* 2010; **66**(6)**:** 1079-85.

38. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL *et al.* Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2012; **30**(5)**:** 488-96.

39. Cramer P, Hallek M. Prognostic factors in chronic lymphocytic leukemia-what do we need to know? *Nature reviews. Clinical oncology* 2011; **8**(1)**:** 38-47.

40. Zaha VG, Young LH. AMP-activated protein kinase regulation and biological actions in the heart. *Circulation research* 2012; **111**(6)**:** 800-14.

41. Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N *et al.* Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature medicine* 2000; **6**(7)**:** 821-5.

42. Tomic J, Lichty B, Spaner DE. Aberrant interferon-signaling is associated with aggressive chronic lymphocytic leukemia. *Blood* 2011; **117**(9)**:** 2668-80.

43. Wong LH, Krauer KG, Hatzinisiriou I, Estcourt MJ, Hersey P, Tam ND *et al.* Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *The Journal of biological chemistry* 1997; **272**(45)**:** 28779-85.

44. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature cell biology* 2011; **13**(2)**:** 132-41.

45. Chiacchiera F, Simone C. Inhibition of p38alpha unveils an AMPK-FoxO3A axis linking autophagy to cancer-specific metabolism. *Autophagy* 2009; **5**(7)**:** 1030-3.

46. Chiacchiera F, Simone C. The AMPK-FoxO3A axis as a target for cancer treatment. *Cell cycle* 2010; **9**(6)**:** 1091-6.

47. Hallek M, Kuhn-Hallek I, Emmerich B. Prognostic factors in chronic lymphocytic leukemia. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 1997; **11 Suppl 2:** S4-13.

48. Hallek M, German CLLSG. Prognostic factors in chronic lymphocytic leukemia. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2008; **19 Suppl 4:** iv51-3.

49. Eichhorst B, Dreyling M, Robak T, Montserrat E, Hallek M, Group EGW. Chronic lymphocytic leukemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2011; **22 Suppl 6:** vi50-4.

50. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell death and differentiation* 2011; **18**(4)**:** 571-80.

CHAPTER 3

2 Discussion

2.1 Limitations in CLL Treatment

With the recent advancements in cancer therapy, there is no curative treatment available for CLL patients. The overall survival rate for CLL patients is fairly low and there is a lot of variability in the clinical progression between patients, making optimal treatment difficult (Brown, 2011). Additionally, many underlying genetic factors impact the response to anti-cancer therapies. Patients who relapse after first-line therapy are a heterogeneous group of patients, making it nearly impossible to pinpoint a central cause for chemoresistance and/or antibody resistance. Up to 40% of CLL patients are refractory to fludarabine. Deletion of 17p-chromosome and mutation of p53 tumor suppressor protein are implicated in this resistance, but other factors are involved as a large percentage of refractory patients do not have these abnormalities (Catovsky *et al.*, 2007; Tsimberidou *et al.*, 2003). It is evident that even following second-line therapies (usually monoclonal antibody-based therapy), CLL can remain refractory (Veliz *et al.*, 2012); thus, novel therapeutic concepts to treat CLL would be beneficial for these patients.

2.2 Oncolytic Virotherapy

Many groups have highlighted the success of oncolytic virotherapy in the treatment of hematological malignancies, including leukemias, lymphomas, multiple myeloma, and the myelodysplastic syndromes (Bais *et al.*, 2012). Studies using VSV show that VSV selectively targets and kills leukemic cells in co-cultures of acute myelogenous leukemia cell lines and normal bone marrow cells (Stojdl *et al.*, 2000). *Ex vivo* primary adult T-cell leukemia (ATL) cells are also sensitive to VSV replication and cytotoxicity (Cesaire *et al.*, 2006). VSV-induced ATL oncolysis is dependent on the

IFN deficiency of cancerous cells, cell cycle progression, and translation initiation in T-lymphocytes (Oliere *et al.*, 2008). Despite having a defective IFN signaling pathway, CLL cells are resistant to VSV infection (Tumilasci *et al.*, 2008). VSV alone can replicate within the cells, but cannot cause viral lysis. CLL cells do not divide and are arrested in the G_0/G_1 phase of the cell cycle; however, this has recently been debated (Messmer *et al.*, 2005). Nonetheless, the resting state of cells may influence sensitivity to the lytic effect of VSV. In spite of the possible role of cell cycle arrest in VSV resistance, dysregulation of apoptosis and autophagy have a greater impact on impaired cell death in CLL.

With the growing interest in Bcl-2 inhibitors to target apoptotic and autophagic pathways for cancer therapy, we decided to design a therapeutic platform where we would use low doses of Bcl-2 inhibitors to enhance VSV-AV1 oncolysis. In Manuscripts I and II, we demonstrated that Bcl-2 inhibitors, Obatoclax, and ABT-737 synergistically enhance cell death in *ex vivo* primary CLL PBMCs infected with VSV-AV1. Combination therapy also reduced tumor burden and prolonged mice survival in an *in vivo* B-lymphoma xenograft tumor model. Even with these promising therapeutic implications, our most significant finding is that combination oncovirotherapy kills *ex vivo* primary CLL PBMCs isolated from refractory patients. Within the cohort of CLL patients studied, we identified numerous prognostic factors with implications in resistance to conventional therapy: CD38 expression, p53, ZAP 70, and IgVH mutation status, and chromosomal abnormalities. Microarray analysis of combination-treated CLL PBMCs revealed similar gene regulation between untreated, refractory, and responder patients following combination treatment. Therefore, Bcl-2 inhibitor/VSV-AV1 combination treatment induces lysis of CLL cells irrespective of their individual genetic characteristics. This "blanket targeting" of Bcl-2 inhibitor/VSV-AV1 therapy makes it an ideal therapeutic candidate for the heterogeneous and multifaceted CLL patient population. This is especially striking because Kojima et al. reported that patients with del[17q] deletions have reduced sensitivity to ABT-737 single treatment (Kojima *et al.*, 2012). Based on our results,

it seems likely that VSV helps overcome this unresponsiveness when combined with ABT-737.

Stem cell transplantation is usually a last resort for CLL therapy. CLL is a disease of the elderly, and many patients are not resilient enough to handle such an aggressive treatment. Autologous hematopoietic stem cell transplantation (ASCT) is often used because of the low mortality rates associated with the treatment and because matching donors are difficult to obtain. A setback of this treatment is the presence of residual, contaminating cancer cells. Because ASCT is normally a last-line therapy, residual cells are normally highly chemoresistant. Recently, encouraging results have been observed with the viral purging of ASCTs. Thirukkumaran et al. have shown that reovirus can effectively purge CLL cells from autografts (Thirukkumaran *et al.*, 2003; Thirukkumaran *et al.*, 2007). Moreover, Stodjl et al. demonstrated similar purging capabilities with VSV therapy in acute myeloid leukemia cells (Stojdl *et al.*, 2000). Collectively, these results suggest that VSV therapy could be a promising approach for ASCT and may reduce the adverse side effects observed with current stem cell transplantation methods.

2.3 Mechanism of Apoptosis Induction

In Manuscript I, we examined the impact of Obatoclax/VSV-AV1 therapy on apoptosis. Wt and mutant forms of VSV activate both extrinsic and intrinsic apoptotic signaling pathways in a cell-type–specific manner (Gaddy *et al.*, 2005, 2007). In Manuscript I, we outlined a detailed mechanism of VSV-mediated apoptotic cell death in CLL cells overexpressing pro-survival proteins. Through disruption of inhibitory pro-survival complexes, Bcl-2-Bax and Mcl-1-Bak, we showed that VSV-AV1-Obatoclax combination therapy activates intrinsic apoptotic signaling. The combination induced cleavage of pro-caspase-3, and -9, whereas the extrinsic apoptotic markers Bid and caspase-8 were not cleaved. Induction of mitochondrial damage was apparent due to the occurrence of Bax translocation and

activation at the mitochondria and cytochrome c release into the cytoplasm. A highlight of this study is the importance of the transcriptional upregulation of Noxa following VSV infection. Noxa is a critical effector of VSV-AV1-Obatoclax–mediated apoptosis. Silencing of Noxa diminishes the apoptotic response to combination therapy, whereas overexpression of Noxa alone, in the absence of VSV infection, promotes cell death. Mechanisms of Noxa-dependent apoptosis have been observed with other OVs. Reovirus infection promotes the upregulation of Noxa in multiple myeloma. In this cancer model, reovirus-enhanced Noxa expression synergistically activates ER stress and apoptosis when combined with anti-cancer drug, bortezomib (Kelly *et al.*, 2012). Oncolytic Sendai virus upregulates Noxa expression in prostate cancer cells (Matsushima-Miyagi *et al.*, 2012). It has also been suggested that Noxa selectively induces death in cancer cells, thereby sparing normal cells from cytotoxicity, although the underlying mechanism for this specificity has not been fully elucidated (Suzuki *et al.*, 2009). Other groups have also stated the importance of Noxa in susceptibility to ABT-737. Absence or low expression levels of Noxa correlates with resistance to ABT-737 treatment in CLL cells, whereas upregulation of Noxa enhanced ABT-737–mediated killing (Tromp *et al.*, 2012). It is apparent that Noxa is critical mediator of CLL death as described in Manuscript I.

Other mechanisms of apoptotic cell death have been described in CLL cells treated with Bcl-2 inhibitor combination therapy. Obatoclax can also synergize with bortezomib to induce death in cancer cells (Perez-Galan *et al.*, 2008). Sensitivity to treatment is dependent of Bcl-2 phosphorylation levels. When Bcl-2 phosphorylation is decreased, CLL cells become more susceptible to Obatoclax therapy. Obatoclax/bortezomib combination therapy disrupted Bcl-2/Bim and Mcl-1/Bak inhibitory complexes in CLL cells. Bcl-2 family regulation is elaborate, and it is evident that countless interactions and protein modifications occur within the cells to inhibit or promote cell death in CLL cells.

2.4 Activation of the Autophagic Pathway

In Manuscript II, we examined the effectiveness of Obatoclax/VSV-AV1 and ABT-737/VSV-AV1 therapy on autophagy. Autophagy has a paradoxical relationship with both cancers and viruses (Orvedahl *et al.*, 2009; Shen *et al.*, 2011; White, 2012). It has been speculated that the inhibition of autophagy by anti-apoptotic Bcl-2 family members directly correlates with tumorigenesis (Pattingre *et al.*, 2006). The mechanism by which autophagy suppresses tumor development is unknown, but it has been proposed that autophagy recycles long-lived proteins and organelles, eliminates damaged organelles that produce reactive oxygen species-mediated genotoxicity, and induces autophagic programmed cell death. From our results, it is apparent that autophagy is dysregulated in CLL cells due to Bcl-2 and, to a certain extent by Mcl-1 overexpression, which obstructs Beclin-1 mediated autophagy. Autophagy may be induced in dying cells in a failed attempt to promote survival. Nevertheless, it is apparent that Bcl-2 inhibitors and VSV-AV1 combination therapy stimulates both the autophagic and apoptotic pathways simultaneously.

At least in terms of OVs, the interrelationship between autophagy and viruses is clearer (Meng *et al.*, 2013). Many oncolytic viruses rely on autophagic processes for viral replication and/or oncolysis. In lung cancer cells, Ad expressing E1b55k proteins (which represses p53-mediated transcription) lyses cancer cells in an autophagy-dependent mechanism. Pharmacological inhibition of autophagy, by 3-MA, can block Ad replication and oncolysis (Rodriguez-Rocha *et al.*, 2011). Ad Delta-24-RGD induces autophagy in human malignant glioma cells (Jiang *et al.*, 2011). Although Delta24RGD viral replication was not dependent on autophagy induction, inhibition of autophagy prevented cell lysis. In this model, autophagy also triggered caspase activity through extrinsic FADD and caspase-8 signaling. This provides new insight into virus-mediated crosstalk between the autophagic and apoptotic signaling pathways. The applicability of the OV/Bcl-2 inhibitor combination treatment to other OVs would depend on the type of virus. Many OVs employ different strategies to induce cell death. Ad dl922-947 replication is inhibited

upon rapamycin-induced activation of autophagy, thus suggesting inhibition of autophagy would potentiate adenovirus dl922-947 induced cell death in contrast to what we observe with VSV (Manuscript II), and other OVs (i.e. Ad Delta24RGD and Ad E1b55k) (Botta *et al.*, 2012). Activation of autophagy as a means to enhance oncolytic virotherapy has emerged as a promising new target for anticancer therapy. Autophagy inducers such as rapamycin and its analogs enhanced VSV-, vaccinia virus-, and Ad-mediated cell death in various cancer model *in vitro* and *in vivo* (Alain *et al.*, 2010; Lun *et al.*, 2009; Taki *et al.*, 2005). Similarly, exploiting the autophagic machinery to augment cancer therapy has also been applied to Bcl-2 inhibitor treatments. Rapamycin in combination with ABT-263 or pan-Bcl-2 inhibitor (−)-gossypol (AT-101) increased the cytotoxic effect of the Bcl-2 inhibitors in follicular lymphoma and glioma cell lines, respectively (Ackler *et al.*, 2008; Voss *et al.*, 2010).Taking these results into consideration, targeting the autophagic process has great potential to optimize both oncolytic virotherapy and Bcl-2 inhibitor therapy, thus expanding potential therapeutic opportunities.

In Manuscript I we demonstrated an essential role for Noxa in VSV mediated apoptosis in *ex vivo* CLL samples. Noxa has a role in autophagy activation as well. Recent reports have shown upregulated Noxa in human ovarian surface epithelial cells disrupts anti-autophagic Mcl-1-Beclin-1 interactions inducing autophagic cell death (Elgendy *et al.*, 2011). Furthermore, Tang et al have suggested a toxic autophagic role with Lapatinib (tyrosine kinase inhibitor) and Obatoclax combination treatment in breast cancer cells that is dependent on Noxa mediated disruption of Mcl-1and Beclin-1(Tang *et al.*, 2012). We demonstrate Beclin-1 release from inhibitory anti-apoptotic proteins, Mcl-1 and Bcl-2, with Obatoclax and ABT-737 inhibitor treatment following VSV viral infection. It is likely that VSV-induced Noxa expression contributes to the disruption of this protein-protein interaction leading to the induction of autophagy in CLL thus maintaining a central role for Noxa in induction of cell death.

In Manuscript II, we also demonstrate that autophagy is regulated by mTOR and FoxO3A, both regulated by AMP-activated protein kinase (AMPK). AMPK is a key cellular energy sensor involved in the regulation of homeostasis (Kahn *et al.*, 2005). AMPK activates a myriad of catabolic pathways in response to cellular stresses (hypoxia, ischemia, and oxidative) that reduce ATP levels and concurrently switches off anabolic pathways (Hardie *et al.*, 2012). mTOR is negatively regulated by AMPK (Chiacchiera *et al.*, 2010; Gwinn *et al.*, 2008). AMPK promotes autophagy through the suppression of mTORC1 or direct phosphorylation of autophagy-initiating kinase Ulk1, preventing its regulation by mTOR (Kalender *et al.*, 2010; Kim *et al.*, 2011). Ulk1 associates with the phagophore during the autophagic process. Forkhead box O (FoxO) member, FoxO3A, is a transcription factor with a role in the suppression of cancer formation (Cornforth *et al.*, 2008; Fernandez de Mattos *et al.*, 2008; Jagani *et al.*, 2008). Phosphorylation of FoxO3a at specific serine and threonine residues results in its inactivation and cytoplasmic targeting (Burgering *et al.*, 2002). AMPK activation targets FoxO3A to the nucleus where the transcription factor accumulates and induces autophagy, cell cycle arrest, and cell death (Chiacchiera *et al.*, 2009). FoxO3A transcript initially induces autophagy as a survival mechanism, but in the presence of prolonged stress, it stimulates autophagic cell death (Chiacchiera *et al.*, 2010).

2.5 Future Perspectives

-

Several OVs have progressed to clinical development in the past few years (Rowan, 2010; Russell *et al.*, 2012). The recent successful results of a phase III trial of talimogene laherparepvec (HSV) for the treatment of metastatic melanoma is a significant milestone in the oncolytic virotherapy field (Amgen, 2013). Talimogene laherparepvec is likely to be the first OV to receive approval in the USA for use in cancer patients (Russell *et al.*, 2012). Oncolytic VSV is being evaluated in an ongoing phase I human clinical trial for the treatment of hepatocellular carcinoma (Hastie *et al.*, 2012). Likewise, numerous Bcl-2 inhibitors are presently being tested

in phase I-III studies, with both Obatoclax and ABT-263 in phase II trials (Azmi *et al.*, 2011). At this point in time, translating OV and Bcl-2 inhibitor combination therapy from pre-clinical studies to the clinic would prove challenging due to the fact that both OVs and Bcl-2 inhibitors are currently being tested independently in human studies. The likelihood of clinically testing the combination at this stage is low as combination product trials usually utilize one or more drugs/agents that have already gained approval.

In Manuscript I and II we observed significant tumor regression and improved animal survival with VSV/Bcl-2 inhibitor treatment in B-lymphoma xenograft mouse models. Nevertheless, pre-clinical murine models do not always predict clinical outcome. Furthermore, CLL is a disseminated disease and administration of OV delivery via the bloodstream can prove challenging. There are many limitations to the therapeutic effectiveness of OV therapy in the clinic (Ferguson *et al.*, 2012; Russell *et al.*, 2012). OVs can be sequestered by liver Kupffer cells and splenic macrophages or can be neutralized by antibodies (Tao *et al.*, 2001; Tsai *et al.*, 2004). Shielding the virus in carrier cells or coating them with polymers counteracts the effects of sequestration and antibody neutralization thus optimizing viral delivery (Fisher *et al.*, 2010; Power *et al.*, 2008; Power *et al.*, 2007). Testing VSV/Bcl-2 inhibitor combination therapy in a CLL animal model such as TCL-1 would be important to further evaluate the efficacy and the safety of the therapy. As in human CLL disease, the B lymphocyte population in TCL-1 mice are CD5+ and expresses Bcl-2 and Mcl-1 protein (Bichi *et al.*, 2002; Johnson *et al.*, 2006). TCL-1 mice also develop enlarged spleens, livers, and lymph nodes. The TCL-1 mouse model may serve as a useful *in vivo* platform to investigate the effects of VSV/Bcl-2 inhibitor treatment for CLL.

OVs can lyse infected cells via several different mechanisms. It would also be of interest to exam other modes of cell death induced by OV/Bcl-2 inhibitor combination treatment including necrosis, ER stress response and the newly emerging necroptosis, as these cell death pathways are linked to both apoptosis and

autophagic cell death (Heath-Engel *et al.*, 2008; Jain *et al.*, 2013; Verfaillie *et al.*, 2010).Various OVs and Bcl-2 inhibitors have been shown to activate or potentiate these signaling pathways leading to cell destruction in cancer cells (Huang *et al.*, 2012; Jiang *et al.*, 2009; Kelly *et al.*, 2012; Petrini *et al.*, 2012). Elucidating alternate mechanisms by which cells undergo death in response to the combination treatment and would provide attractive new targets for cancer therapy.

References

Ackler, S., Xiao, Y., Mitten, M. J., Foster, K., Oleksijew, A., Refici, M., Schlessinger, S., Wang, B., Chemburkar, S. R., Bauch, J., Tse, C., Frost, D. J., Fesik, S. W., Rosenberg, S. H., Elmore, S. W., & Shoemaker, A. R. (2008). ABT-263 and rapamycin act cooperatively to kill lymphoma cells in vitro and in vivo. *Mol Cancer Ther, 7*(10), 3265-3274. doi: 10.1158/1535-7163.MCT-08-0268

Alain, T., Lun, X., Martineau, Y., Sean, P., Pulendran, B., Petroulakis, E., Zemp, F. J., Lemay, C. G., Roy, D., Bell, J. C., Thomas, G., Kozma, S. C., Forsyth, P. A., Costa-Mattioli, M., & Sonenberg, N. (2010). Vesicular stomatitis virus oncolysis is potentiated by impairing mTORC1-dependent type I IFN production. *Proc Natl Acad Sci U S A, 107*(4), 1576-1581. doi: 10.1073/pnas.0912344107

Amgen. (2013). Amgen Announces Top-Line Results Of Phase 3 Talimogene Laherparepvec Trial In Melanoma [News Release]. Retrieved from http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1798143

Azmi, A. S., Wang, Z., Philip, P. A., Mohammad, R. M., & Sarkar, F. H. (2011). Emerging Bcl-2 inhibitors for the treatment of cancer. *Expert Opin Emerg Drugs, 16*(1), 59-70. doi: 10.1517/14728214.2010.515210

Bais, S., Bartee, E., Rahman, M. M., McFadden, G., & Cogle, C. R. (2012). Oncolytic virotherapy for hematological malignancies. *Adv Virol, 2012*, 186512. doi: 10.1155/2012/186512

Bajwa, N., Liao, C., & Nikolovska-Coleska, Z. (2012). Inhibitors of the anti-apoptotic Bcl-2 proteins: a patent review. *Expert Opin Ther Pat, 22*(1), 37-55. doi: 10.1517/13543776.2012.644274

Barber, G. N. (2004). Vesicular stomatitis virus as an oncolytic vector. *Viral Immunol, 17*(4), 516-527. doi: 10.1089/vim.2004.17.516

Bichi, R., Shinton, S. A., Martin, E. S., Koval, A., Calin, G. A., Cesari, R., Russo, G., Hardy, R. R., & Croce, C. M. (2002). Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci U S A, 99*(10), 6955-6960. doi: 10.1073/pnas.102181599

Botta, G., Passaro, C., Libertini, S., Abagnale, A., Barbato, S., Maione, A. S., Hallden, G., Beguinot, F., Formisano, P., & Portella, G. (2012). Inhibition of autophagy enhances the effects of E1A-defective oncolytic adenovirus dl922-947 against glioma cells in vitro and in vivo. *Hum Gene Ther, 23*(6), 623-634. doi: 10.1089/hum.2011.120

Breitbach, C. J., Burke, J., Jonker, D., Stephenson, J., Haas, A. R., Chow, L. Q., Nieva, J., Hwang, T. H., Moon, A., Patt, R., Pelusio, A., Le Boeuf, F., Burns, J., Evgin, L., De Silva, N., Cvancic, S., Robertson, T., Je, J. E., Lee, Y. S., Parato, K., Diallo, J. S., Fenster, A., Daneshmand, M., Bell, J. C., & Kirn, D. H. (2011). Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature, 477*(7362), 99-102. doi: 10.1038/nature10358

Brown, J. R. (2011). The treatment of relapsed refractory chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program, 2011*, 110-118. doi: 10.1182/asheducation-2011.1.110

Brunelle, J. K., & Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci, 122*(Pt 4), 437-441. doi: 10.1242/jcs.031682

Buggins, A. G., & Pepper, C. J. (2010). The role of Bcl-2 family proteins in chronic lymphocytic leukaemia. *Leuk Res, 34*(7), 837-842. doi: 10.1016/j.leukres.2010.03.011

Burgering, B. M., & Kops, G. J. (2002). Cell cycle and death control: long live Forkheads. *Trends Biochem Sci, 27*(7), 352-360.

Campas, C., Cosialls, A. M., Barragan, M., Iglesias-Serret, D., Santidrian, A. F., Coll-Mulet, L., de Frias, M., Domingo, A., Pons, G., & Gil, J. (2006). Bcl-2 inhibitors induce apoptosis in chronic lymphocytic leukemia cells. *Exp Hematol, 34*(12), 1663-1669. doi: 10.1016/j.exphem.2006.07.008

Catovsky, D., Richards, S., Matutes, E., Oscier, D., Dyer, M. J., Bezares, R. F., Pettitt, A. R., Hamblin, T., Milligan, D. W., Child, J. A., Hamilton, M. S., Dearden, C. E., Smith, A. G., Bosanquet, A. G., Davis, Z., Brito-Babapulle, V., Else, M., Wade, R., Hillmen, P., Group, U. K. National Cancer Research Institute Haematological Oncology Clinical Studies, & Group, Ncri Chronic Lymphocytic Leukaemia Working. (2007). Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. *Lancet, 370*(9583), 230-239. doi: 10.1016/S0140-6736(07)61125-8

Cesaire, R., Oliere, S., Sharif-Askari, E., Loignon, M., Lezin, A., Olindo, S., Panelatti, G., Kazanji, M., Aloyz, R., Panasci, L., Bell, J. C., & Hiscott, J. (2006). Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia. *Oncogene, 25*(3), 349-358. doi: 10.1038/sj.onc.1209055

Chiacchiera, F., & Simone, C. (2009). Inhibition of p38alpha unveils an AMPK-FoxO3A axis linking autophagy to cancer-specific metabolism. *Autophagy, 5*(7), 1030-1033.

Chiacchiera, F., & Simone, C. (2010). The AMPK-FoxO3A axis as a target for cancer treatment. *Cell Cycle, 9*(6), 1091-1096.

Chiorazzi, N., Rai, K. R., & Ferrarini, M. (2005). Chronic lymphocytic leukemia. *N Engl J Med, 352*(8), 804-815. doi: 10.1056/NEJMra041720

Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J., & Green, D. R. (2010). The BCL-2 family reunion. *Mol Cell, 37*(3), 299-310. doi: 10.1016/j.molcel.2010.01.025

Cornforth, A. N., Davis, J. S., Khanifar, E., Nastiuk, K. L., & Krolewski, J. J. (2008). FOXO3a mediates the androgen-dependent regulation of FLIP and contributes to TRAIL-induced apoptosis of LNCaP cells. *Oncogene, 27*(32), 4422-4433. doi: 10.1038/onc.2008.80

Cramer, P., & Hallek, M. (2011). Prognostic factors in chronic lymphocytic leukemia-what do we need to know? *Nat Rev Clin Oncol, 8*(1), 38-47. doi: 10.1038/nrclinonc.2010.167

Donnelly, O. G., Errington-Mais, F., Prestwich, R., Harrington, K., Pandha, H., Vile, R., & Melcher, A. A. (2012). Recent clinical experience with oncolytic viruses. *Curr Pharm Biotechnol, 13*(9), 1834-1841.

Eager, R. M., & Nemunaitis, J. (2011). Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther, 18*(5), 305-317. doi: 10.1038/cgt.2011.7

Eichhorst, B., Dreyling, M., Robak, T., Montserrat, E., Hallek, M., & Group, Esmo Guidelines Working. (2011). Chronic lymphocytic leukemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol, 22 Suppl 6*, vi50-54. doi: 10.1093/annonc/mdr377

Elgendy, M., Sheridan, C., Brumatti, G., & Martin, S. J. (2011). Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. *Mol Cell, 42*(1), 23-35. doi: 10.1016/j.molcel.2011.02.009

Ferguson, M. S., Lemoine, N. R., & Wang, Y. (2012). Systemic delivery of oncolytic viruses: hopes and hurdles. *Adv Virol, 2012*, 805629. doi: 10.1155/2012/805629

Fernandez de Mattos, S., Villalonga, P., Clardy, J., & Lam, E. W. (2008). FOXO3a mediates the cytotoxic effects of cisplatin in colon cancer cells. *Mol Cancer Ther, 7*(10), 3237-3246. doi: 10.1158/1535-7163.MCT-08-0398

Fisher, K. D., & Seymour, L. W. (2010). HPMA copolymers for masking and retargeting of therapeutic viruses. *Adv Drug Deliv Rev, 62*(2), 240-245. doi: 10.1016/j.addr.2009.12.003

Gaddy, D. F., & Lyles, D. S. (2005). Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. *J Virol, 79*(7), 4170-4179. doi: 10.1128/JVI.79.7.4170-4179.2005

Gaddy, D. F., & Lyles, D. S. (2007). Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx. *J Virol, 81*(6), 2792-2804. doi: 10.1128/JVI.01760-06

Gandhi, L., Camidge, D. R., Ribeiro de Oliveira, M., Bonomi, P., Gandara, D., Khaira, D., Hann, C. L., McKeegan, E. M., Litvinovich, E., Hemken, P. M., Dive, C., Enschede, S. H., Nolan, C., Chiu, Y. L., Busman, T., Xiong, H., Krivoshik, A. P., Humerickhouse, R., Shapiro, G. I., & Rudin, C. M. (2011). Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. *J Clin Oncol, 29*(7), 909-916. doi: 10.1200/JCO.2010.31.6208

Garcia-Saez, A. J. (2012). The secrets of the Bcl-2 family. *Cell Death Differ, 19*(11), 1733-1740. doi: 10.1038/cdd.2012.105

Gordy, C., & He, Y. W. (2012). The crosstalk between autophagy and apoptosis: where does this lead? *Protein Cell, 3*(1), 17-27. doi: 10.1007/s13238-011-1127-x

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E., & Shaw, R. J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell, 30*(2), 214-226. doi: 10.1016/j.molcel.2008.03.003

Hallek, M., Cheson, B. D., Catovsky, D., Caligaris-Cappio, F., Dighiero, G., Dohner, H., Hillmen, P., Keating, M. J., Montserrat, E., Rai, K. R., Kipps, T. J., &

International Workshop on Chronic Lymphocytic, Leukemia. (2008). Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood, 111*(12), 5446-5456. doi: 10.1182/blood-2007-06-093906

Hallek, M., & German, C. L. L. Study Group. (2008). Prognostic factors in chronic lymphocytic leukemia. *Ann Oncol, 19 Suppl 4*, iv51-53. doi: 10.1093/annonc/mdn196

Hallek, M., Kuhn-Hallek, I., & Emmerich, B. (1997). Prognostic factors in chronic lymphocytic leukemia. *Leukemia, 11 Suppl 2*, S4-13.

Hardie, D. G., Ross, F. A., & Hawley, S. A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol, 13*(4), 251-262. doi: 10.1038/nrm3311

Hastie, E., & Grdzelishvili, V. Z. (2012). Vesicular stomatitis virus as a flexible platform for oncolytic virotherapy against cancer. *J Gen Virol, 93*(Pt 12), 2529-2545. doi: 10.1099/vir.0.046672-0

Heath-Engel, H. M., Chang, N. C., & Shore, G. C. (2008). The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. *Oncogene, 27*(50), 6419-6433. doi: 10.1038/onc.2008.309

High, L. M., Szymanska, B., Wilczynska-Kalak, U., Barber, N., O'Brien, R., Khaw, S. L., Vikstrom, I. B., Roberts, A. W., & Lock, R. B. (2010). The Bcl-2 homology domain 3 mimetic ABT-737 targets the apoptotic machinery in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interactions with established drugs. *Mol Pharmacol, 77*(3), 483-494. doi: 10.1124/mol.109.060780

Hou, W., Han, J., Lu, C., Goldstein, L. A., & Rabinowich, H. (2010). Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. *Autophagy, 6*(7), 891-900. doi: 10.4161/auto.6.7.13038

Huang, H., Xiao, T., He, L., Ji, H., & Liu, X. Y. (2012). Interferon-beta-armed oncolytic adenovirus induces both apoptosis and necroptosis in cancer cells. *Acta Biochim Biophys Sin (Shanghai), 44*(9), 737-745. doi: 10.1093/abbs/gms060

Jagani, Z., Singh, A., & Khosravi-Far, R. (2008). FoxO tumor suppressors and BCR-ABL-induced leukemia: a matter of evasion of apoptosis. *Biochim Biophys Acta, 1785*(1), 63-84. doi: 10.1016/j.bbcan.2007.10.003

Jain, M. V., Paczulla, A. M., Klonisch, T., Dimgba, F. N., Rao, S. B., Roberg, K., Schweizer, F., Lengerke, C., Davoodpour, P., Palicharla, V. R., Maddika, S., & Los, M. (2013). Interconnections between apoptotic, autophagic and necrotic pathways: implications for cancer therapy development. *J Cell Mol Med, 17*(1), 12-29. doi: 10.1111/jcmm.12001

Jiang, C. C., Wroblewski, D., Yang, F., Hersey, P., & Zhang, X. D. (2009). Human melanoma cells under endoplasmic reticulum stress are more susceptible to apoptosis induced by the BH3 mimetic obatoclax. *Neoplasia, 11*(9), 945-955.

Jiang, H., White, E. J., Rios-Vicil, C. I., Xu, J., Gomez-Manzano, C., & Fueyo, J. (2011). Human adenovirus type 5 induces cell lysis through autophagy and

autophagy-triggered caspase activity. *J Virol, 85*(10), 4720-4729. doi: 10.1128/JVI.02032-10

Johnson, A. J., Lucas, D. M., Muthusamy, N., Smith, L. L., Edwards, R. B., De Lay, M. D., Croce, C. M., Grever, M. R., & Byrd, J. C. (2006). Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia. *Blood, 108*(4), 1334-1338. doi: 10.1182/blood-2005-12-011213

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab, 1*(1), 15-25. doi: 10.1016/j.cmet.2004.12.003

Kalender, A., Selvaraj, A., Kim, S. Y., Gulati, P., Brule, S., Viollet, B., Kemp, B. E., Bardeesy, N., Dennis, P., Schlager, J. J., Marette, A., Kozma, S. C., & Thomas, G. (2010). Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab, 11*(5), 390-401. doi: 10.1016/j.cmet.2010.03.014

Kang, R., Zeh, H. J., Lotze, M. T., & Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ, 18*(4), 571-580. doi: 10.1038/cdd.2010.191

Kelly, K. R., Espitia, C. M., Mahalingam, D., Oyajobi, B. O., Coffey, M., Giles, F. J., Carew, J. S., & Nawrocki, S. T. (2012). Reovirus therapy stimulates endoplasmic reticular stress, NOXA induction, and augments bortezomib-mediated apoptosis in multiple myeloma. *Oncogene, 31*(25), 3023-3038. doi: 10.1038/onc.2011.478

Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol, 13*(2), 132-141. doi: 10.1038/ncb2152

Kojima, K., Duvvuri, S., Ruvolo, V., Samaniego, F., Younes, A., & Andreeff, M. (2012). Decreased sensitivity of 17p-deleted chronic lymphocytic leukemia cells to a small molecule BCL-2 antagonist ABT-737. *Cancer, 118*(4), 1023-1031. doi: 10.1002/cncr.26360

Levine, B., Sinha, S., & Kroemer, G. (2008). Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy, 4*(5), 600-606.

Lichty, B. D., Power, A. T., Stojdl, D. F., & Bell, J. C. (2004). Vesicular stomatitis virus: re-inventing the bullet. *Trends Mol Med, 10*(5), 210-216. doi: 10.1016/j.molmed.2004.03.003

Lichty, B. D., Stojdl, D. F., Taylor, R. A., Miller, L., Frenkel, I., Atkins, H., & Bell, J. C. (2004). Vesicular stomatitis virus: a potential therapeutic virus for the treatment of hematologic malignancy. *Hum Gene Ther, 15*(9), 821-831. doi: 10.1089/hum.2004.15.821

Lun, X. Q., Jang, J. H., Tang, N., Deng, H., Head, R., Bell, J. C., Stojdl, D. F., Nutt, C. L., Senger, D. L., Forsyth, P. A., & McCart, J. A. (2009). Efficacy of systemically administered oncolytic vaccinia virotherapy for malignant gliomas is enhanced by combination therapy with rapamycin or cyclophosphamide. *Clin Cancer Res, 15*(8), 2777-2788. doi: 10.1158/1078-0432.CCR-08-2342

Mason, K. D., Khaw, S. L., Rayeroux, K. C., Chew, E., Lee, E. F., Fairlie, W. D., Grigg, A. P., Seymour, J. F., Szer, J., Huang, D. C., & Roberts, A. W. (2009). The BH3 mimetic compound, ABT-737, synergizes with a range of cytotoxic chemotherapy agents in chronic lymphocytic leukemia. *Leukemia, 23*(11), 2034-2041. doi: 10.1038/leu.2009.151

Matsushima-Miyagi, T., Hatano, K., Nomura, M., Li-Wen, L., Nishikawa, T., Saga, K., Shimbo, T., & Kaneda, Y. (2012). TRAIL and Noxa are selectively upregulated in prostate cancer cells downstream of the RIG-I/MAVS signaling pathway by nonreplicating Sendai virus particles. *Clin Cancer Res, 18*(22), 6271-6283. doi: 10.1158/1078-0432.CCR-12-1595

Meng, S., Xu, J., Wu, Y., & Ding, C. (2013). Targeting autophagy to enhance oncolytic virus-based cancer therapy. *Expert Opin Biol Ther, 13*(6), 863-873. doi: 10.1517/14712598.2013.774365

Messmer, B. T., Messmer, D., Allen, S. L., Kolitz, J. E., Kudalkar, P., Cesar, D., Murphy, E. J., Koduru, P., Ferrarini, M., Zupo, S., Cutrona, G., Damle, R. N., Wasil, T., Rai, K. R., Hellerstein, M. K., & Chiorazzi, N. (2005). In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest, 115*(3), 755-764. doi: 10.1172/JCI23409

Nguyen, M., Marcellus, R. C., Roulston, A., Watson, M., Serfass, L., Murthy Madiraju, S. R., Goulet, D., Viallet, J., Belec, L., Billot, X., Acoca, S., Purisima, E., Wiegmans, A., Cluse, L., Johnstone, R. W., Beauparlant, P., & Shore, G. C. (2007). Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A, 104*(49), 19512-19517. doi: 10.1073/pnas.0709443104

Oliere, S., Arguello, M., Mesplede, T., Tumilasci, V., Nakhaei, P., Stojdl, D., Sonenberg, N., Bell, J., & Hiscott, J. (2008). Vesicular stomatitis virus oncolysis of T lymphocytes requires cell cycle entry and translation initiation. *J Virol, 82*(12), 5735-5749. doi: 10.1128/JVI.02601-07

Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., & Rosenberg, S. H. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature, 435*(7042), 677-681. doi: 10.1038/nature03579

Orvedahl, A., & Levine, B. (2009). Autophagy in Mammalian antiviral immunity. *Curr Top Microbiol Immunol, 335*, 267-285. doi: 10.1007/978-3-642-00302-8_13

Paik, P. K., Rudin, C. M., Brown, A., Rizvi, N. A., Takebe, N., Travis, W., James, L., Ginsberg, M. S., Juergens, R., Markus, S., Tyson, L., Subzwari, S., Kris, M. G., & Krug, L. M. (2010). A phase I study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in solid tumor malignancies. *Cancer Chemother Pharmacol, 66*(6), 1079-1085. doi: 10.1007/s00280-010-1265-5

Pandey, R., Chander, R., & Sainis, K. B. (2009). Prodigiosins as anti cancer agents: living upto their name. *Curr Pharm Des, 15*(7), 732-741.

Pattingre, S., & Levine, B. (2006). Bcl-2 inhibition of autophagy: a new route to cancer? *Cancer Res, 66*(6), 2885-2888. doi: 10.1158/0008-5472.CAN-05-4412

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D., & Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell, 122*(6), 927-939. doi: 10.1016/j.cell.2005.07.002

Perez-Galan, P., Roue, G., Lopez-Guerra, M., Nguyen, M., Villamor, N., Montserrat, E., Shore, G. C., Campo, E., & Colomer, D. (2008). BCL-2 phosphorylation modulates sensitivity to the BH3 mimetic GX15-070 (Obatoclax) and reduces its synergistic interaction with bortezomib in chronic lymphocytic leukemia cells. *Leukemia, 22*(9), 1712-1720. doi: 10.1038/leu.2008.175

Petrini, I., Meltzer, P. S., Zucali, P. A., Luo, J., Lee, C., Santoro, A., Lee, H. S., Killian, K. J., Wang, Y., Tsokos, M., Roncalli, M., Steinberg, S. M., Wang, Y., & Giaccone, G. (2012). Copy number aberrations of BCL2 and CDKN2A/B identified by array-CGH in thymic epithelial tumors. *Cell Death Dis, 3*, e351. doi: 10.1038/cddis.2012.92

Power, A. T., & Bell, J. C. (2008). Taming the Trojan horse: optimizing dynamic carrier cell/oncolytic virus systems for cancer biotherapy. *Gene Ther, 15*(10), 772-779. doi: 10.1038/gt.2008.40

Power, A. T., Wang, J., Falls, T. J., Paterson, J. M., Parato, K. A., Lichty, B. D., Stojdl, D. F., Forsyth, P. A., Atkins, H., & Bell, J. C. (2007). Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. *Mol Ther, 15*(1), 123-130. doi: 10.1038/sj.mt.6300039

Roberts, A. W., Seymour, J. F., Brown, J. R., Wierda, W. G., Kipps, T. J., Khaw, S. L., Carney, D. A., He, S. Z., Huang, D. C., Xiong, H., Cui, Y., Busman, T. A., McKeegan, E. M., Krivoshik, A. P., Enschede, S. H., & Humerickhouse, R. (2012). Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol, 30*(5), 488-496. doi: 10.1200/JCO.2011.34.7898

Rodriguez-Rocha, H., Gomez-Gutierrez, J. G., Garcia-Garcia, A., Rao, X. M., Chen, L., McMasters, K. M., & Zhou, H. S. (2011). Adenoviruses induce autophagy to promote virus replication and oncolysis. *Virology, 416*(1-2), 9-15. doi: 10.1016/j.virol.2011.04.017

Rowan, K. (2010). Oncolytic viruses move forward in clinical trials. *J Natl Cancer Inst, 102*(9), 590-595. doi: 10.1093/jnci/djq165

Russell, S. J., Peng, K. W., & Bell, J. C. (2012). Oncolytic virotherapy. *Nat Biotechnol, 30*(7), 658-670. doi: 10.1038/nbt.2287

Samuel, S., Tumilasci, V. F., Oliere, S., Nguyen, T. L., Shamy, A., Bell, J., & Hiscott, J. (2010). VSV oncolysis in combination with the BCL-2 inhibitor obatoclax overcomes apoptosis resistance in chronic lymphocytic leukemia. *Mol Ther, 18*(12), 2094-2103. doi: 10.1038/mt.2010.188

Shanshal, M., & Haddad, R. Y. (2012). Chronic lymphocytic leukemia. *Dis Mon, 58*(4), 153-167. doi: 10.1016/j.disamonth.2012.01.009

Shen, H. M., & Codogno, P. (2011). Autophagic cell death: Loch Ness monster or endangered species? *Autophagy, 7*(5), 457-465.

Sinha, S., & Levine, B. (2008). The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene, 27 Suppl 1*, S137-148. doi: 10.1038/onc.2009.51

Stojdl, D. F., Lichty, B. D., tenOever, B. R., Paterson, J. M., Power, A. T., Knowles, S., Marius, R., Reynard, J., Poliquin, L., Atkins, H., Brown, E. G., Durbin, R. K., Durbin, J. E., Hiscott, J., & Bell, J. C. (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell, 4*(4), 263-275.

Stojdl, D. F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N., & Bell, J. C. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med, 6*(7), 821-825. doi: 10.1038/77558

Suzuki, S., Nakasato, M., Shibue, T., Koshima, I., & Taniguchi, T. (2009). Therapeutic potential of proapoptotic molecule Noxa in the selective elimination of tumor cells. *Cancer Sci, 100*(4), 759-769. doi: 10.1111/j.1349-7006.2009.01096.x

Taki, M., Kagawa, S., Nishizaki, M., Mizuguchi, H., Hayakawa, T., Kyo, S., Nagai, K., Urata, Y., Tanaka, N., & Fujiwara, T. (2005). Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD'). *Oncogene, 24*(19), 3130-3140. doi: 10.1038/sj.onc.1208460

Tang, Y., Hamed, H. A., Cruickshanks, N., Fisher, P. B., Grant, S., & Dent, P. (2012). Obatoclax and lapatinib interact to induce toxic autophagy through NOXA. *Mol Pharmacol, 81*(4), 527-540. doi: 10.1124/mol.111.076851

Tao, N., Gao, G. P., Parr, M., Johnston, J., Baradet, T., Wilson, J. M., Barsoum, J., & Fawell, S. E. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther, 3*(1), 28-35. doi: 10.1006/mthe.2000.0227

Thirukkumaran, C. M., Luider, J. M., Stewart, D. A., Cheng, T., Lupichuk, S. M., Nodwell, M. J., Russell, J. A., Auer, I. A., & Morris, D. G. (2003). Reovirus oncolysis as a novel purging strategy for autologous stem cell transplantation. *Blood, 102*(1), 377-387. doi: 10.1182/blood-2002-08-2508

Thirukkumaran, C. M., Russell, J. A., Stewart, D. A., & Morris, D. G. (2007). Viral purging of haematological autografts: should we sneeze on the graft? *Bone Marrow Transplant, 40*(1), 1-12. doi: 10.1038/sj.bmt.1705668

Tomic, J., Lichty, B., & Spaner, D. E. (2011). Aberrant interferon-signaling is associated with aggressive chronic lymphocytic leukemia. *Blood, 117*(9), 2668-2680. doi: 10.1182/blood-2010-05-285999

Townsend, K. N., Hughson, L. R., Schlie, K., Poon, V. I., Westerback, A., & Lum, J. J. (2012). Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. *Immunol Rev, 249*(1), 176-194. doi: 10.1111/j.1600-065X.2012.01141.x

Tromp, J. M., Geest, C. R., Breij, E. C., Elias, J. A., van Laar, J., Luijks, D. M., Kater, A. P., Beaumont, T., van Oers, M. H., & Eldering, E. (2012). Tipping the Noxa/Mcl-1 balance overcomes ABT-737 resistance in chronic lymphocytic leukemia. *Clin Cancer Res, 18*(2), 487-498. doi: 10.1158/1078-0432.CCR-11-1440

Tsai, V., Johnson, D. E., Rahman, A., Wen, S. F., LaFace, D., Philopena, J., Nery, J., Zepeda, M., Maneval, D. C., Demers, G. W., & Ralston, R. (2004). Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model. *Clin Cancer Res, 10*(21), 7199-7206. doi: 10.1158/1078-0432.CCR-04-0765

Tse, C., Shoemaker, A. R., Adickes, J., Anderson, M. G., Chen, J., Jin, S., Johnson, E. F., Marsh, K. C., Mitten, M. J., Nimmer, P., Roberts, L., Tahir, S. K., Xiao, Y., Yang, X., Zhang, H., Fesik, S., Rosenberg, S. H., & Elmore, S. W. (2008). ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res, 68*(9), 3421-3428. doi: 10.1158/0008-5472.CAN-07-5836

Tsimberidou, A. M., Kantarjian, H. M., Cortes, J., Thomas, D. A., Faderl, S., Garcia-Manero, G., Verstovsek, S., Ferrajoli, A., Wierda, W., Alvarado, Y., O'Brien, S. M., Albitar, M., Keating, M. J., & Giles, F. J. (2003). Fractionated cyclophosphamide, vincristine, liposomal daunorubicin, and dexamethasone plus rituximab and granulocyte-macrophage-colony stimulating factor (GM-CSF) alternating with methotrexate and cytarabine plus rituximab and GM-CSF in patients with Richter syndrome or fludarabine-refractory chronic lymphocytic leukemia. *Cancer, 97*(7), 1711-1720. doi: 10.1002/cncr.11238

Tumilasci, V. F., Oliere, S., Nguyen, T. L., Shamy, A., Bell, J., & Hiscott, J. (2008). Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary chronic lymphocytic leukemia cells to vesicular stomatitis virus-induced oncolysis. *J Virol, 82*(17), 8487-8499. doi: 10.1128/JVI.00851-08

Veliz, M., & Pinilla-Ibarz, J. (2012). Treatment of relapsed or refractory chronic lymphocytic leukemia. *Cancer Control, 19*(1), 37-53.

Verfaillie, T., Salazar, M., Velasco, G., & Agostinis, P. (2010). Linking ER Stress to Autophagy: Potential Implications for Cancer Therapy. *Int J Cell Biol, 2010*, 930509. doi: 10.1155/2010/930509

Voss, V., Senft, C., Lang, V., Ronellenfitsch, M. W., Steinbach, J. P., Seifert, V., & Kogel, D. (2010). The pan-Bcl-2 inhibitor (-)-gossypol triggers autophagic cell death in malignant glioma. *Mol Cancer Res, 8*(7), 1002-1016. doi: 10.1158/1541-7786.MCR-09-0562

White, E. (2012). Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer, 12*(6), 401-410. doi: 10.1038/nrc3262

Wong, L. H., Krauer, K. G., Hatzinisiriou, I., Estcourt, M. J., Hersey, P., Tam, N. D., Edmondson, S., Devenish, R. J., & Ralph, S. J. (1997). Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *J Biol Chem, 272*(45), 28779-28785.

Zaha, V. G., & Young, L. H. (2012). AMP-activated protein kinase regulation and biological actions in the heart. *Circ Res, 111*(6), 800-814. doi: 10.1161/CIRCRESAHA.111.255505

Zhou, F., Yang, Y., & Xing, D. (2011). Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *FEBS J, 278*(3), 403-413. doi: 10.1111/j.1742-4658.2010.07965.x

CHAPTER 4

Concluding Remarks and Contribution to Original Knowledge

Oncolytic virotherapies represent an exciting new biological approach to cancer therapy and are relatively safe and less toxic than current standard cancer treatments (i.e. chemotherapy and radiotherapy). The combination of VSV-AV1 and Bcl-2 inhibitors have important implications for the clinical development of well tolerated and effective combination therapies for many cancers, particularly those with similar mechanisms of deregulated cell death to that of CLL, including other leukemias, prostate and breast cancers.

Therapeutic targeting of apoptosis-signaling pathways has been well established for several cancer models. The autophagic pathway, however, has recently emerged as a prime candidate for the identification of novel targets for drug development and the improvement of current cancer therapies; nevertheless, some controversy remains regarding the link between autophagy and cancer. Identifying the principal modes of impaired cell death (autophagy and apoptosis) in various cancer types will facilitate the identification of the most appropriate and specific treatment for cancer patients.

In the studies presented within, we showed that the autophagic and apoptotic pathways are both dysregulated in CLL. Using a novel and safe (minimal effects on healthy and non-leukemic cells) combination therapy approach, we combined VSV-AV1 and Bcl-2 antagonists and synergistically induced cell killing in CLL cells *ex vivo*. Strikingly, this synergism was observed in primary CLL cells isolated from patients known to be resistant to conventional CLL therapies. The implications of such a treatment are substantial, especially because very low doses of Bcl-2 inhibitor were required to have a synergistic effect with oncolytic virotherapy. This is important because Bcl-2 inhibitors are known to have dose-limiting complications in

clinical trials. Moreover, our studies elucidated the mechanism of apoptosis and autophagy-associated oncolysis mediated by Bcl-2 inhibitor/VSV-AV1 virotherapy. Our results clearly show that the combination therapy induced apoptosis through transcriptional upregulation of Noxa, the abrogation Bcl-2/Bax, and Mcl-1/Bak complexes, and the cleavage of intrinsic mitochondrial caspases-3 and -9 **(**Figure 1). In parallel, Bcl-2 inhibitor/VSV-AV1 treatment stimulated autophagy and displaced Beclin-1 from anti-apoptotic Bcl-2 proteins, induced autophagosome formation (LC3 cleavage), enhanced autophagic flux (diminished p62 levels), and initiated mTOR and FOXO3A dependent regulation of autophagy. Essentially, these results provide a significantly more comprehensive view of the apoptotic and autophagic processes involved in VSV-dependent oncolysis.

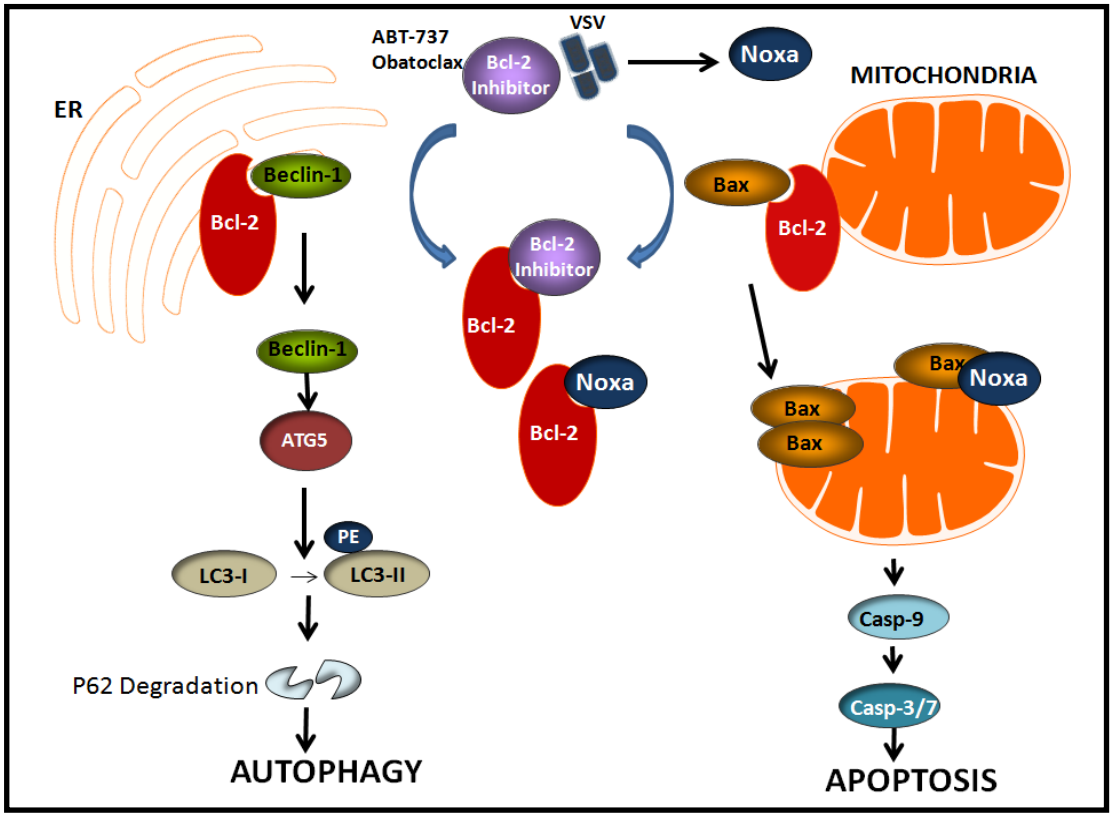

Figure 1: Summary of the findings presented in this thesis.

In CLL cells, Bcl-2 inhibitors, Obatoclax (GX15-070) and ABT-737 occupy the BH-3-binding groove of pro-survival Bcl-2 proteins disrupting protein-protein interactions with Bax/Bak and restore intrinsic apoptotic signaling in response to VSV infection (right). VSV induces the transcriptional upregulation of NOXA which can also displace pro-survival interactions and/or directly activate Bax. Released Bax/Bak can oligomerize and permeabilize the mitochondrial membrane inducing cytochrome c release and caspase activation. During autophagy (left) Bcl-2 inhibitor-VSV combination disrupts anti-autophagic Bcl-2 interactions with Beclin-1.When Beclin-1 is released, downstream proteins necessary for autophagosome formation such as Atg5 and LC3-II are activated and p62, a determinant of autophagic flux (the equilibrium between autophagosome formation and clearance by lysosomes), is degraded. **BCL-2:** B-cell lymphoma-2; **VSV:** vesicular stomatitis virus, **LC3**: Microtubule-associated protein light chain 3; **Atg5**: Autophagy protein 5