Improving VSV virotherapy in Chronic Lymphocytic Leukemia with small-molecule BCL-2 inhibitors

Vanessa Fonseca Tumilasci

Department of Microbiology and Immunology, Faculty of Medicine

McGill University, Montreal

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ABSTRACT

Oncolytic virus therapy is a new form of cancer treatment that uses viruses that preferentially infect and lyse cancer cells. Vesicular stomatitis virus (VSV) is a strong oncolytic virus candidate that infects multiple tumor cells, produces rapid viral replication in malignant cells and spreads quickly in the tumor. Defects in the interferon (IFN) antiviral pathway are common in tumor cells and such defects are accountable for the sensitivity to VSV infection and replication in several malignant cells. The intrinsic mitochondrial apoptotic pathway plays a crucial role in VSV-induced apoptosis and disturbance of this pathway is responsible for resistance to VSV oncolysis of cancer cells. The antiapoptotic protein B-cell lymphoma 2 (BCL-2) is commonly overexpressed in tumor cells, especially in hematological malignancies, and is strongly related to resistance to cancer therapy. Chronic lymphocytic leukemia (CLL) is an accumulative disease of mature-looking $CD5^+/CD19^+$ lymphocytes, caused by defects in apoptosis rather than increase in proliferation. CLL patients express high levels of the BCL-2 protein which correlates with poor treatment outcome. Small-molecule BCL-2 inhibitors showed promising anticancer properties in preclinical models. A phase I clinical trial demonstrated modest activity against CLL. Based on our observation that CLL cells are resistant to VSV-induced cell death due to overexpression of BCL-2, we hypothesized that inhibition of BCL-2 could restore VSV oncolytic potential in primary CLL cells. In fact, BCL-2 inhibitors EM20-25

and obatoclax sensitized primary CLL cells to VSV-induced cell death. Mechanistically, while VSV infection triggered Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) upregulation, obatoclax blocked the ability of BCL-2 to dimerize with the proapoptotic BCL2-associated X protein (BAX). Together, NOXA expression and BAX release were able to efficiently induce apoptosis. Moreover, our data demonstrated a direct interaction between NOXA and BAX. Together, these data indicate that the use of BCL-2 inhibitors may improve VSV oncolysis in apoptosis-resistant hematological malignancies characterized by overexpression of anti-apoptotic proteins such as BCL-2.

RESUMÉ

La Virothérapie est une nouvelle forme de traitement pour le cancer qui utilise des virus qui infectent préférentiellement et lysent les cellules cancéreuses. Le virus de la stomatite vésiculaire (VSV) est un candidat solide à virus oncolytique, il infecte les cellules de tumeurs, il produit la réplication virale rapide dans les cellules malignes et se propage rapidement au sein de la tumeur. Irrégularités dans la voie antivirale d'interféron (IFN) sont communes dans les cellules tumorales et en virothérapie avec VSV, ces défauts sont responsables pour la sensibilité générale à l'infection pour VSV et sa réplication dans les cellules cancéreuses. La voie apoptotique intrinsèque à mitochondrie joue un rôle crucial dans l'apoptose induite par VSV et la perturbation de cette voie est responsable pour la résistance à la virothérapie avec VSV. La protéine anti-apoptotique B-cell lymphoma 2 (BCL-2) est fréquemment surexprimé dans les cellules tumorales, en particulier dans les hémopathies malignes, et est fortement liée à la résistance au traitement du cancer. La leucémie lymphoïde chronique (CLL) est une maladie d'accumulation de lymphocytes $CD5^{+}/CD19^{+}$, causée par une apoptose défectueuse plutôt qui une prolifération accrue. Les patients CLL présentent souvent des niveaux très élevés de la protéine BCL-2 qui se traduisent dans de pauvres résultats de traitement. Petites molécules inhibitrices du BCL-2 ont montré de propriétés anticancéreuses prometteuses dans des modèles précliniques, mais les essais cliniques de phase I ont démontré une activité modeste contre

CLL. Notre observation que les cellules de CLL sont résistantes à l'induction de la mort cellulaire par VSV due à la surexpression de BCL-2 nous a menée à emmètre l'hypothèse que l'inhibition de BCL-2 pourrait rétablir le potentiel oncolytique du VSV dans les cellules primaires de CLL. En effet, les inhibiteurs de BCL-2 EM20-25 et obatoclax ont sensibilisé les cellules CLL *ex vivo* à la mort cellulaire induite par VSV. Ainsi, l'infection avec VSV a déclenché la régulation de NOXA à la hausse est obatoclax a bloqué la capacité de BCL-2 à dimerizer avec la protéine proapoptotique BAX. Ensemble, l'expression de NOXA et la libération de BAX étaient capable d'induire efficacement l'apoptose. De plus, nos données démontrent une interaction directe entre NOXA et BAX. Ensemble, ces données indiquent que l'utilisation des inhibiteurs de BCL-2 pourront améliorer la résistance à l'apoptose induite par VSV surtout dans les hémopathies malignes caractérisées par une surexpression de protéines anti-apoptotiques tels que BCL-2.

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Many people have helped me throughout these almost 6 years and have contributed positively to make this work complete; I would like to take this opportunity to thank them:

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Over the last years, I feel that I have obtained a unique experience both professionally and personally that I will be able to carry with me in my future endeavors.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis preparation from the Faculty of Graduate Studies and Research of McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. For this, the guidelines state: "…Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. …..The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. ….The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. ….In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. …..In addition, the candidate is

required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. ….." As chapters of this thesis, I have included the texts and figures of two original research manuscripts that have been published (Chapter 2 and Chapter 3). Each of these chapters contains its own summary, introduction, materials and methods, results and discussion. In addition, a preface is included at the beginning of each chapter in order to introduce and bridge the papers with connecting texts. A general introduction and literature review is presented in Chapter 1, and a final discussion is included in Chapter 4. The references for chapters 1 through 4 are included at the end of this thesis.

The manuscripts included in this thesis are as follows:

Chapter 2: **Tumilasci VF**, Olière S, Nguyên 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 Sep;82(17):8487-99.

Chapter 3: Samuel S, **Tumilasci VF**, Oliere S, Nguyên TLA, Shamy A, Bell J and Hiscott J. VSV oncolysis in combination with the BCL-2 inhibitor obatoclax

overcomes apoptosis resistance in chronic lymphocytic leukemia. Mol Ther; 2010, 18:2094-2103.

STATEMENT OF ORIGINALITY

The body of work described in this thesis represents original research by the PhD student, Vanessa Fonseca Tumilasci. I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material and has not been presented previously nor has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

CONTRIBUTIONS OF AUTHORS

The candidate performed the majority of the research presented in this thesis. The candidate's research and the thesis supervisor, Dr. John Hiscott, provided scientific direction to all data produced. Dr. Hiscott assisted in the conception of experiments and in the writing of 2 original research manuscripts described in this dissertation.

The specific contribution of authors to this work is described as follows:

Chapter 2: **Tumilasci VF**, Olière S, Nguyên 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 Sep;82(17):8487-99.

Tumilasci VF: All the experimental work, data analysis and manuscript writing.

Olière S: Contributed with PBMCs isolation and FACS analysis.

Nguyên TL: Assisted with discussion regarding oncolytic viruses experiments and manuscript editing.

Shamy A: Selection of CLL patients.

Bell J: Provided VSV strains used in this work.

Hiscott J.: Manuscript editing and study supervision.

Chapter 3: Samuel S*, **Tumilasci VF***, Oliere S, Nguyên TL, Shamy A, Bell J and Hiscott J. VSV oncolysis in combination with the BCL-2 inhibitor obatoclax overcomes apoptosis resistance in chronic lymphocytic leukemia. Mol Ther (2010); Sep 14. * these authors contributed equally to this work

Vanessa Fonseca Tumilasci: performed and designed the experiments in Figures 1A, 1B, 2A, 2B, 3A, 3C, 7A, 7B and 7C. The candidate designed the other experiments with Sara Samuel. The candidate was also responsible for data analysis and interpretation of the data as presented in this thesis. The candidate wrote the drafts of the manuscript as presented herein.

Sara Samuel: carried out the experiments in all other figures with the exception of Figure 2C, manuscript correction and editing.

Stephanie Oliere: Animal experiment execution.

Nguyên TL: Assisted with discussion regarding oncolytic viruses' experiments and manuscript editing.

April Shamy: Selection of CLL patients.

John Bell: Provided VSV strains used in this work.

John Hiscott: Manuscript editing and study supervision.

CONTRIBUTIONS NOT INCLUDED IN THIS THESIS

In addition to the manuscripts included in this thesis, the candidate contributed to the following published studies and reviews:

Tumilasci VF, Nguyen TL, Singhroy D, Arguello M, Hiscott J. The emergence of combinatorial strategies in the development of RNA oncolytic virus therapies. Cell Microbiol. 2009 Jun;11(6):889-97.

Oliere S, Arguello M, Mesplede T, **Tumilasci V**, Nakhaei P, Stojdl D, Sonenberg N, Bell J, Hiscott J. Vesicular stomatitis virus oncolysis of T lymphocytes requires cell cycle entry and translation initiation. J. Virol. 2008 Jun;82(12):5735-49.

Lemay G, **Tumilasci V**, Hiscott J. Uncoating reo: uncovering the steps critical for oncolysis. Mol Ther. 2007 Aug;15(8):1406-7

Holm GH, **Tumilasci V**, Zurney J , Leveille S, Danthi P, Hiscott J, Sherry B, Dermody TS. Retinoic acid-inducible gene-I and interferon-beta promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3. J Biol Chem. 2007 Jul 27;282(30):21953-61

Sharif-Askari E, Nakhaei P, Oliere S, **Tumilasci V**, Hernandez E, Wilkinson P, Lin R, Bell J, Hiscott J. Bax-dependent mitochondrial membrane permeabilization enhances IRF3-mediated innate immune response during VSV infection. Virology. 2007;365(1):20-33.

Peyman Nakhaei, Suzanne Paz, Stephanie Oliere, **Vanessa Tumilasci**, John C. Bell and John Hiscott. Oncolytic virotherapy of cancer with vesicular stomatitis virus. Gene Therapy and Molecular Biology 2005; 9:269.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A1: BCL-2-related protein A1

Ad: Adenovirus

AIM2: Absent in melanoma 2 protein

AKT: Serine/threonine protein kinase (protein kinase B)

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

APAF-1: apoptotic protease-activating factor-1

ASOs: Antisense oligonucleotides

BAD: BCL-2-associated death promoter

BAK: BCL-2 homologous antagonist/killer

BAX: BCL-2–associated X protein

BCL-2: B-cell lymphoma 2

BCL-w: BCL-2-like protein 2

BCL-xL: B-cell lymphoma-extra large protein

BH: BCL-2 homology domain

BID: BH3 interacting domain death agonist

BIK: BCL-2 Interacting Killer

BIM: BCL-2-like protein 11

BLK: BH3-containing mouse protein

BOD: BCLL-2-Related Ovarian Death Gene

BOK: BCL2-related ovarian killer

cADPR: Cyclic ADP-ribose

c-FLIP: FLICE inhibitory protein

CAR: Coxsackie-adenovirus receptor

CARDs: RIG-I-like receptor (RLR) family

CBP: CREB binding protein

CLB: Chlorambucil

CEA: Carcinoembryonic antigen

CI: Combination index

CLL: Chronic Lymphocytic Leukemia

CML: Chronic myeloid leukemia

CNS: Central Nervous System

CPE: Cytopathic effects

CpG: 2′-deoxyribo(cytidinephosphateguanosine) DNA motifs

CREB: cAMP-responsive element binding protein

CTX: Cyclophosphamide

DAMPs: Danger or damage associated molecular patterns

DCs: Dendritic cells

DD: Death Domain

DFF40/CAD: 40-kDa caspase-3-activated nuclease/Caspase-activated DNase

DFF45/ICAD: DNA fragmentation factor 45/inhibitor of caspase activated DNAse

DISC: Death-induced signaling complexes

DNA: Deoxyribonucleic acid

DR3: Death Receptor 3

DR6: Death Receptor 6

dsRNA: Double-stranded RNA

ERK: Elk-related tyrosine kinase

ETF: Early transcription factor

EGFR: Epidermal growth factor receptor

ERK: Elk-related tyrosine kinase

F : Fusion protein

FACS: Fluorescence Activated Cell Sorting

FADD: FAS-associated death domain-containing protein

FAS: TNF receptor superfamily, member 6

FASL: FAS ligand protein

FC: fludarabine and cyclophosphamide regimen

FCR: fludarabine, rituximab and cyclophosphamide

GBM: Glioblastoma multiforme

GM-CSF: Granulocyte macrophage colony-stimulator factor

GX15-070: Obatoclax mesylate

HDIs: Histone deacetylase inhibitors

HERC5: Hect domain and RLD5

HN: Hemagglutinin-neuraminidase

HRK: Activator of apoptosis harakiri

HSP70: Heat Shock Protein 70

HSV: Herpes Simplex Virus

IFN: Interferon

IFNAR: Type I IFN receptor

IgVH: immunoglobulin heavy locus

IKK-α: Ikappa B kinase alpha

IKK-ε: IkappaB kinase-epsilon

IL: Interleukin

IL-1R: Interleukin-1-receptor

IP: Intraperitoneal

IRAK1 : Interleukin-1-receptor-associated kinase-1

IRAK4 : Interleukin-1-receptor-associated kinase-4

IRF-3: Interferon regulatory factor 3

IRF-7: Interferon regulatory factor 7

IRF-9: Interferon regulatory factor 9

ISGs: Interferon stimulated genes

ISGF3: IFN-stimulated gene factor 3

ISREs: IFN-stimulated response elements

ISVPs: Reovirus infectious subvirion particles

IWCLL: International Workshop on CLL

JAM-A: Junctional adhesion molecule-A

JAK-1: Tyrosine kinase 1

JNK: Janus kinase

L: Large polymerase protein

LGP2: Laboratory of genetics and physiology 2 protein

M : Matrix protein

MAMPs: Microbial associated molecular patterns

MAVS: Mitochondrial anti-viral signaling (MAVS) adaptor protein

MCL: Mantle cell lymphoma

MCL-1: Myeloid leukemia cell differentiation protein

MDA-5: Melanoma differentiation-associated gene 5

MDS: Myelodysplastic syndrome

miRs: microRNAs

MM: Multiple myeloma

MOMP: Mitochondrial outer membrane permeabilization

MTT: Tetrazolium dye

MV: Measles Virus

NCI-WG: National Cancer Institute Working Group on CLL

ND: Newcastle Disease

NDV: Newcastle Disease Virus

NEMO: NF-κB essential modulator

NF-κB: Nuclear factor-KappaB

NHL: Human follicular non-Hodgkin lymphoma

NK: Natural killer cells

NLRs: Nucleotide-binding domain, leucine-rich repeat

NOXA: PMA Phorbol-12-myristate-13-acetate-induced protein 1

NP : Nucleocapsid protein

NPC: Nuclear pore complex

NSCLC: Non-small cell lung carcinoma

OVs: Oncolytic Viruses

P : phosphoprotein

PAMPs: Pathogen associated molecular patterns

PARP: Poly (ADP ribose) polymerase

PBMCs: Peripheral blood mononuclear cells

PGRs: Peptidoglycan recognition proteins

PKR: double stranded RNA-dependent protein kinase

PRRs: Pattern recognition receptors

PS: Phosphatidylserine

REOVIRUS: Respiratory Enteric Orphan Virus

RIG-I: Retinoic acid inducible gene I

RLRs: RIG-I-like receptor family

RNA: Ribonucleic acid

RR: Ribonucleotide reductase

SCLC: Small cell lung carcinoma

siRNA: Small-interfering RNA

SLAM: Signaling lymphocytic activation molecule

SLL: Small lymphocytic leukemia

ssRNA: Single-stranded RNA

STAT: Signal Transducer and Activator of Transcription

STING: Stimulator of interferon genes

Syk: spleen tyrosine kinase

T1L: Reovirus type 1 Lang strain

T2J : Reovirus type 2 Jones strain

T2W : Reovirus type 2 Winnipeg strain

T3D : Reovirus type 3 Dearing strain

TBK1: TANK-binding kinase 1

TK: Thymidine kinase

TH1: T helper type 1 cells

TH2: T helper type 2 cells

TIR: Toll-interleukin 1 receptor

TLRs: Toll-like receptors

TNF: Tumor necrosis factor

TNFR1: TNF receptor-1

TRADD: TNF receptor 1-associated protein

TRAF3: Tumor necrosis factor-receptor associated factor 3

TRAF6: Tumor necrosis factor-receptor associated factor 6

TRAIL: Tumor necrosis factor (ligand) superfamily, member 10

TRAILR1: TRAIL Receptor 1

TRAILR2: TRAIL Receptor 2

TYK2: Tyrosine kinase 2

VSV: Vesicular Stomatitis Virus

VV: Vaccinia virus

ZAP-70: Zeta-associated protein with the molecular weight of 70 kD

LIST OF TABLES AND FIGURES

CHAPTER 1

CHAPTER 2

INTRODUCTION

Toxicity, development of resistance, and limited efficacy of existing cancer therapies have provided the impetus to search for new approaches [1-4]. Virotherapy is one new approach to cancer treatment that has gained importance during the last decade. Virotherapy uses oncolytic viruses (OVs), which have the capacity to replicate inside cancer cells and induce cell death, while sparing normal cells [5-7]. Specificity of oncolytic viruses is achieved by exploiting intracellular defects that develop during tumor evolution, such as the RAS signaling pathway and the interferon (IFN) antiviral pathway [8-13].

Vesicular stomatitis virus (VSV) is an enveloped, negative-stranded RNA virus with a genome encoding five proteins [14-15]. Among the naturally occurring strains of VSV, two variants – AV1 and AV2, which contain mutations in the M protein – are considered to be natural oncolytic viruses, with increased oncolytic capacity compared to wild-type VSV [11, 16-17]. VSV has strong sensitivity to the IFN antiviral response [11, 13, 18-22], and deficiencies in the interferon response, which are present in many cancer cells [11, 23-24], enhance susceptibility of tumor cell lines and *ex vivo* cancer cells to VSV oncolysis. To date, VSV has proven to be effective against many types of cancer, including prostate cancer [25], gliomas [18], colon cancer [26], and leukemia [27-28]. VSV induces cell death by activating programmed cell death (apoptosis) [29-32]. Both intrinsic and extrinsic apoptotic pathways have been correlated with VSV-induced

apoptosis, but the involvement of mitochondria appears to be crucial for VSV-induced cell death.

Chronic lymphocytic leukemia (CLL), one of the most prevalent forms of human leukemia, is characterized by accumulation of the abnormal $CD5^+/CD19^+$ subset of lymphocytes in blood and bone marrow [33-34]. The primary pathogenic event that causes the generation of aberrant B cells remains to be determined, but impaired apoptosis results in increased survival of circulating CLL cells. High levels of prosurvival proteins can be detected in CLL cells [35-36] and are associated with resistance to apoptosis and chemotherapy [37-38]. Although VSV oncolysis has been reported in different leukemic models [27-28], CLL is resistant to VSV-induced cell death [27, 39]. VSV-induced apoptosis requires a functional mitochondrial apoptotic pathway, and defects in the intrinsic apoptotic pathway are related to impaired activation of apoptosis in cancer cells [40-44]. If resistance of CLL cells to VSV-oncolysis is explained by the presence of high levels of B-cell lymphoma 2 (BCL-2) anti-apoptotic protein, strategies that restore the mitochondrial apoptotic pathway might be successfully used in combination with VSV virotherapy.

Small molecule BCL-2 antagonists, such as obatoclax, ABT-737, and HA14-1 are a new category of anticancer agents with promising results against resistant leukemias [45-49]. Small-molecule BCL-2 inhibitors prevent the binding of anti-

apoptotic proteins to the pro-apoptotic counterpart [50-53], promoting apoptosis in malignant cells [54-57]. Small-peptide BCL-2 antagonists are being tested in Phase I/II clinical trials for various leukemia, lymphoma, and solid tumor malignancies, both as single agents and in combination therapies [58-59].

The present study investigated the use of small-molecule BCL-2 inhibitors to overcome CLL resistance to VSV-induced oncolysis. The research had three specific goals. The first was to evaluate the potential of small-molecule BCL-2 inhibitors to improve VSV virotherapy, using cell lines overexpressing the BCL-2 protein and *ex vivo* CLL cells. Primary peripheral blood mononuclear cells (PBMCs), isolated from healthy volunteers, were used as controls. The second goal was to elucidate the molecular mechanism involved in apoptosis induced by the combination of VSV oncolytic virus and BCL-2 inhibitors, using B-cells with high levels of the BCL-2 protein. Involvement of the intrinsic mitochondrial apoptotic pathway was evaluated by monitoring release of cytochrome *c* into the cytoplasm and activation of effector caspases. BAX and NOXA proteins are closely associated with VSV-induced apoptosis [29, 60-61]. These proteins were followed to evaluate interactions with anti-apoptotic proteins, such as BCL-2 and MCL-1. The third goal was to evaluate the efficacy of combined VSV and BCL-2 inhibitors in an *in vivo* xenograft model, specifically, the A20 mouse B-lymphocyte cell line in SCID mice.

LITERATURE REVIEW

Oncolytic viruses

Viruses, either naturally selected or genetically engineered, that are used as treatment against cancer are called oncolytic viruses (OVs). The ability of viruses to kill tumors is not a new concept. More than a century ago, scientists reported the use of live viruses in cancer patients, particularly those with leukemia [62-65]. Early in the twentieth century, cases of cancer regression in patients with natural viral infections were reported; later, clinical responses were noted in cancer patients that received vaccinations for viruses, such as vaccinia and measles. The viruses replicated and lysed tumors, often in immunosupressed patients. Clinical trials of Newcastle disease virus (NDV) [66] and Reovirus [67] in 2006 confirmed their oncolytic properties.

The most significant characteristic of OVs is their ability to replicate within cancer cells, destroying them while sparing normal cells (Table 1) [5-7, 11, 68-69]. Evolution of cancer cells result in extensive point mutations and chromosomal instability, which ultimately leads to uncontrolled proliferation [6, 70-73]. These molecular alterations also created defects in important defense signaling pathways, which allow OVs to replicate within cancer cells and trigger cell death. For example, the ability of Herpes simplex virus (HSV) to infect transformed NIH-3T3 cells, but not their untransformed counterparts, is related to the RAS and double stranded RNA-dependent protein kinase (PKR) pathways

[12]. Cells with a functional PKR pathway and low activity in the rat sarcoma viral oncogene (RAS) pathway are not permissive to HSV-1, while cells with strong anti-PKR activity are permissive. dsRNA produced during viral replication is sensed by PKR which is expressed ubiquitously at low levels as an inactive monomer [74]. Binding of dsRNA to PKR alters the conformation of PKR, which leads to activation of the kinase by dimerization and autophosphorylation. Activated PKR phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2α) which converts eIF2 to a competitive inhibitor of eIF2B function [74]. Inhibition of eIF2B activity decreases translation initiation and thus aborting both cellular metabolism and viral replication. The oncolytic properties of Reovirus are also associated with activation of the RAS pathway: replication is possible in RAS-transformed cells and dependent on RalGEF signaling [8]. RAS proteins are membrane-bound molecules that regulate a wide range of cellular functions, including differentiation, proliferation and cell survival. Tumor cells bearing an activated RAS pathway and RAS-transformed cells are deficient in their ability to activate the anti-viral response mediated by the host cellular protein, PKR [75]. Activation of RAS protein stimulates several effector proteins such as phosphatidylinositol 3- kinase (PI3-K) and RAF and RAL guanine nucleotide-dissociation stimulators (RALGDS) to regulate cell proliferation and survival [75]. In particular, activated RAF kinase activates the mitogen-activated protein kinase (MAPK) cascade and a critical downstream RAS effector MAPK kinase,

the MEK kinase, has been associated with PKR inhibition via MEK-induced ERK1 and ERK2 phosphorylation – the Ras/Raf/MEK/ERK pathway [76].

The interferon (IFN) pathway also plays an important role in the selectivity of OVs for cancer cells. Infection of normal cells with Vesicular stomatitis virus (VSV) triggers activation of the IFN pathway, conferring high protection to normal tissue against viral infection. A large number of cancer cell lines have defects in the IFN pathway, allowing VSV to infect, spread, and kill cells in a variety of tumor tissue [9, 11, 13].

Among viruses used as OVs, RNA viruses, such as VSV and Reovirus, possess natural tumor-tropism, which enables them to exclusively replicate in cells with defective antiviral response systems with no engineering. Other potential OVs, including Adenovirus and Vaccinia virus require recombination and engineering to achieve tumor cell selectivity (Table 1).

Newcastle disease virus (NDV), which belongs to the Paramyxoviridae family, is an enveloped, non-segmented, negative-sense single-stranded (ss) RNA virus. Its 15Kb genome encodes six proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase protein (L) [77]. NDV causes Newcastle disease (ND), a contagious disease in birds that causes economic problems in the poultry industry

worldwide [78]. ND is characterized by respiratory and central nervous system problems, such as coughing, sneezing, gasping for air, nasal discharge, and paralysis of the legs, wings or neck, tremors, and drooping wings. NDV is typically non-pathogenic in humans, but rare neuropathogenicity occurs in the form of meningitis or encephalitis, with full recovery.

NDV has been used as an experimental oncolytic agent for more than 30 years [79]. It enters the cell through attachment of hemagglutinin-neuraminidase (HN) to sialic acid receptors, such as signaling lymphocyte-activating molecules (SLAM) [80-81]. Once the HN viral protein is attached to the receptor, viral fusion (F) protein mediates fusion of viral and cell membranes, which allow release of viral RNA into the cell cytoplasm, where replication takes place. The F protein undergoes proteolytic processing: its precursor, F0, is cleaved at a specific cleavage site by a furin-like protease and becomes a metastable form. Two subunits, F1 and F2, become bonded by disulfide bridges and produce the active form of the protein [80-81]. The amino acid sequence of the F0 protein cleavage site is responsible for virulence of the virus [82-84]. The amino acid sequence, ^{112}R-R-Q-K/R-R^{117}, is required for efficient cleavage of F0 protein of velogenic viruses (highly virulent viruses), and mesogenic viruses (moderately virulent). This sequence is identical to the consensus amino acid sequence, ^{112}R/K-R-Q-R/K-R-F^{117}, of virulent NDV strains. Selective infection of human tumor cells by NDV is dependent on production of danger signals that are identifiable by retinoic

acid inducible gene I (RIG-I) and double stranded RNA-dependent protein kinase (PKR) in the cytoplasm, and by toll-like receptors (TLRs) in endosomes [85-86]. These pathways lead to immune cell activation through production of antiviral cytokines, such as interferon (IFN). Naturally-attenuated strains of NDV are highly sensitive to the IFN response; therefore, defects in the IFN pathway are thought to allow NDV infection of malignant cells.

NDV infection stimulates immune responses by triggering T-cell-, macrophage-, and NK cell-mediated responses and cytokine production. IFN alpha (IFNα) is released during the course of NDV infection, and activates dendritic and NK cells [87-89]. Active NK cells and cytotoxic T-cells kill virus-infected tumor cells by release of cytoplasmic granules from intracellular organelles (i.e Perforin and Granzyme B). Perforin mediate induction of apoptosis by forming pores in the cell membrane of the target cell, creating a channel through which the Granzyme B can enter the cell and induce apoptosis. Granzyme B cleaves caspase-3 which activates caspase-activated DNAse, an enzyme that degrades DNA. Granzyme B can also induce Bid cleavage which activates the intrinsic mitochondrial pathway. NK cells can also kill virus-infected tumor cells through death ligands that belong to the tumor necrosis factor (TNF)-family. Cell death can be achieved by production of infection-induced tumor necrosis factor alpha (TNFα) which can activate NK cells, macrophages and sensitized T-cells. TNFα upregulates expression of the TNF-related apoptosis-inducing ligand (TRAIL) receptors on

the surface of infected cells, inducing activation of the extrinsic apoptotic pathway [77, 90]. NDV infection also elicits activation of the intrinsic mitochondrial apoptotic pathway through release of cytochrome *c* and caspase-9 activation. Activation of the mitochondrial apoptotic pathway by NDV has been recently demonstrated to involve BAX activation and translocation to the mitochondria membrane [91-92]. The ability of p53 to transcriptionally activate BAX has been demonstrated [93]. Ravindra *et al* demonstrated that NDV infection causes significant up-regulation in p53 expression that coincides with increase BAX/BCL-2 ratio [92]. The Mitochondrial antiviral signaling (MAVS, also known as IPS-1, VISA or Cardif) is an important adaptor protein in viral sensing in the cytoplasm and it is implicated MAVS in type 1 IFN responses. Localization of MAVS in the mitochondria led researchers to investigate putative function of MAVS in apoptosis. Recently, MAVS has been demonstrated to induce apoptosis independently of IFN-I although the mechanism of MAVS-induced apoptosis has yet to be discovered [94]. However, the role of MAVS in cell death induction in response to NDV infection remains unclear.

Naturally attenuated NDV strains have consistently produced promising results, when used as vaccines in clinical studies [66, 95]. Clinical trials were conducted to evaluate vaccines of attenuated NDV: 73-T and PV701 strains were tested for treatment of advanced solid tumors, and Ulster and NDV-HUJ strains were tested for treatment of glioblastoma multiforme (GBM). A Phase I clinical trial was

designed to characterize the toxicity profile of prolonged, repeated intravenous PV701 dosing, and the results showed no cumulative toxicity [96]. An intensification of more than 100-fold caused only flu-like symptoms, usually after the first dose, which decreased in number and severity with each subsequent dose. Thirty-nine patients received 116 repeat courses, and one patient received more than 30 courses of PV701, with no evidence of an adverse effect on any organ system. A latter clinical trial with PV701, tested whether two-step desensitization, using two dose increments before high repeat doses, would be well tolerated [97]. The results demonstrated no dose-limiting toxicities. Flu-like symptoms were observed after first infusion and diminished with repeated dosing. Tumor regression was observed in one patient and four patients had disease stabilization for 6 months. A Phase I/II study was designed to determine the safety and tumor response of repetitive intravenous administration of NDV-HUJ in recurrent GBM [66]. NDV-HUJ was well tolerated, and complete response was achieved in one patient. Adverse reactions were observed in five of eleven patients, usually during the first cycle. A recently initiated clinical trial will evaluate intravenous treatment with the NDV-HUJ strain in patients with advanced GBM, soft and bone sarcomas, and neuroblastomas (http://clinicaltrials.gov ID: NCT01174537). All of these cancers previously proved resistant to conventional anti-cancer modalities. The promising results from NDV in pre-clinical studies and, most importantly, the encouraging observations from clinical trials indicate that this virus merits further study as an agent for cancer treatment.

Respiratory enteric orphan (REO) virus, or **Reovirus**, is a double-stranded (ds) RNA virus belonging to the Reoviridae family, with a segmented genome of ten individual linear dsRNA molecules. Four reovirus serotypes have been identified. Type 1 Lang (T1L), type 2 Jones (T2J) and type 2 Winnipeg (T2W), and type 3 Dearing (T3D) can be differentiated by the ability of anti-sera to neutralize viral infectivity and to inhibit hemagglutination [98-100]. A putative fourth serotype of reovirus, Ndelle virus, has also been reported.

Reovirus causes mild infections of the upper respiratory and gastrointestinal tract of humans with no clinical significance [101-102]. Reovirus enters the host by either respiratory or enteric routes and infects the epithelium and associated lymphoid tissue. To enter a cell, the virus must attach to receptors on the cell surface. The main receptor for entry is the junctional adhesion molecule-A (JAM-A), which is bound by the viral σ1 protein [103-104]. JAM-A is a tight junction protein, and is an important feature of the endothelial cells that line blood vessels and lymphatic vessels. Therefore, JAM-A is important to infection through the hematogenous route [105]. Once attached to the receptor, internalization of the virus occurs via receptor-mediated endocytosis in clathrin vesicles [106]. Exposure of the virus to low pH and to endosomal and lysosomal proteases activates membrane penetration and degrades the viral σ3 protein [107-110]. σ3, the outermost capsid protein, is digested by proteases, mainly cathepsins, leaving

the capsid protein, μ1, exposed on the particle surface. Exposure of μ1 forms infectious subvirion particles (ISVPs). Reovirus ISVPs induce membrane permeabilization and conversion of ISVP to ISVP*, which involves conformational changes in the μ1 protein, specifically, protease-induced C-terminal cleavage and an autocatalytic N-terminal [111-112]. One of the resulting fragments, μ1N, forms size-selective pores in the membrane and allows the ISVPs to enter the cytoplasm, where they transcribe parental genome RNA into mRNA.

Reovirus particles contain a complete enzyme system, which transcribes the ten capped genome segments, but no polyadenylated segments [113-115]. Because their genome is a double-stranded RNA, it cannot function as mRNA; therefore, reoviruses contain an RNA polymerase to make mRNA after infection of the host cell. The RNA polymerase (or transcriptase) catalyzes mRNA synthesis in which positive strand transcripts from each of the ten dsRNA segments are synthesized, capped, and methylated by virion packaged enzymes. The transcripts are accumulated in distinct regions of the cytoplasm (viral factories) and used as templates for translation [114-115]. The resulting proteins are assembled, forming immature capsids in which the mRNAs are copied to form new dsRNA. The virus is released from the cytoplasm by lysis of the cell, not by budding.

Reoviruses are tumor selective, due to their ability to replicate in the presence of an activated RAS pathway. The virus cannot block the antiviral effect of PKR,

and depends on activation of the RAS pathway to induce an endogenous protein inhibitor of PKR [102, 116-117]. Approximately 15-30% of human tumors possess an activating mutation of the RAS pathway. Pancreatic and colon cancers have the highest prevalence of an activating mutation (90 and 50%, respectively) [75, 118-119]. Pre-clinical studies have shown that reovirus can accomplish tumor regression in solid malignancies and hematopoietic tumors [102, 120-122]. Furthermore, clinical trials have demonstrated that reovirus is a safe and powerful oncolytic agent [116-117]. Reolysin®, developed by Oncolytics Biotech Inc. (Calgary, AB, Canada) from naturally occurring reovirus, has been used in Phase I and II clinical trials with over 300 patients (www.clinicaltrials.gov; www.oncolyticsbiotech.com/clinical.html). The results indicate that reovirus is likely to have activity across a broad range of tumor types and is well-tolerated by patients. Clinical responses have been observed with single-agent reovirus, and current studies are investigating the efficacy of reovirus in combination with other treatment modalities across a broad range of tumor types.

Measles virus (MV) is the causative agent of measles, a disease usually acquired in childhood and characterized by high fever and a skin rash [123]. Measles remains a leading cause of death among young children, with almost 200,000 deaths worldwide in 2007 (http://www.who.int). MV is an enveloped, non-segmented, negative sense, ssRNA virus that belongs to the Paramyxoviridae family [123-124]. It has a genome of 15.9 Kb, which encodes six structural

proteins [nucleocapsid (N), phosphoprotein (P), matrix (M), large polymerase (L), the ribonucleoprotein particle (RNP)-complex, and the envelope protein] and two non-structural proteins [hemagglutinin (HN) and fusion (F)].

MV enters the human body through the respiratory tract, where it replicates. From the respiratory tract, it penetrates the lymphatic system, enters the blood, and ultimately spreads throughout the body. Cell penetration occurs via virus-cell membrane fusion, which is triggered by virus attachment to host cell receptors present at the cell surface, and by F protein-promoted fusion [124]. CD46, a cell surface type 1 transmembrane glycoprotein, ubiquitously expressed in all nucleated human cells, was reported to act as cellular receptor to MV [124-126]. CD150, the SLAM membrane glycoprotein, which is expressed on various cells of the immune system, was also identified as an MV receptor [124, 127]. MV attaches to the receptor on the host cell surface by binding with the HN protein, activating membrane fusion [128-129]. MV fusion protein is present in the virus as the non-fusogenic precursor form, F0. Once fusion process is activated, F0 is proteolytically processed by furin-like protein at the RRFKR site into a heterodimer, F1+F2. The heterodimer primes the protein for fusion by positioning the fusion peptide at the N-terminus of F1. The fusion peptide is inserted into the host cell membrane, initiating membrane fusion, which allows uptake of the viral core complex into the host cell cytoplasm [130]. The viral fusion process may also cause fusion of the infected cell with adjacent non-infected cells (syncytia

formation), if the adjacent cell expresses the appropriate receptors for the hemagglutinin protein [131].

Once fusion occurs, the nucleocapsid is released into the cytoplasm, where its acts as a template for primary transcription of mRNA, and replication of the negative-stranded genome RNA into positive-stranded RNA [129, 131]. Viral mRNAs are then capped, methylated, and polyadenylated and the resulting RNA strands are immediately coated with nucleocapsid protein. During viral assembly, H and F proteins are translated as transmembrane proteins and transported to the cell plasma membrane, where they interact with the nucleocapsids through a process enabled by the M protein. As a final step of viral replication, the virus buds out through the cell membrane.

MV was first isolated in 1954, from a patient named David Edmonston. This strain was attenuated by multiple culture passages, and the resulting attenuated Edmonston strain is used worldwide for vaccination [132]. The potential of the attenuated Edmonston strain as an oncolytic viral agent was discovered in 2004 [132]. Tropism of MV for tumor cells, especially its lymphotropism, might be explained by the expression of the SLAM receptor on the cells of the immune system [133]. CD46 is expressed in all nucleated cells, which accounts for MV infection in other cell types [123, 134-135]. These two molecules are commonly overexpressed in tumor cells, which make malignant cells a desirable target for

MV. When MV infects tumor cells, non-viable multinucleated structures, syncytia, are developed which generate a bystander killing effect [136-137]. Expression of the viral hemagglutinin and fusion proteins on the infected cell membranes stimulates fusion with uninfected surrounding cells. Upregulation of heat shock protein 70 (HSP70) is induced in cells infected with MV, and may contribute to the cytopathic effects [137].

The oncolytic ability of MV has been confirmed in several murine models, including lymphoma, myeloma, ovarian carcinoma, and glioma [123, 138]. In a Phase I clinical trial, MV was engineered to express the marker peptide, carcinoembryonic antigen (CEA) resulting in the MV-CEA strain [139]. Insertion of this peptide allowed quantitative monitoring of viral gene expression. The trial was conducted on 21 patients with platinum-refractory recurrent ovarian cancer and normal CEA levels, who were also treated with Taxol. The results showed no dose-limiting toxicity, treatment-induced immunosuppression, increase in anti-MV antibody titers and virus shedding in urine or saliva. CEA levels were increased in peritoneal fluid and serum, confirming viral replication. The study demonstrated that intraperitoneal (IP) administration of MV-CEA is well tolerated, and dose-dependent disease stabilization was achieved in 67% of patients. Two Phase I clinical trials were started in 2009 ([140]; www. http://clinicaltrials.gov). One trial is evaluating side effects of MV-CEA and determining the optimal dose of viral therapy for patients with recurrent

glioblastoma multiforme. The second trial is evaluating MV-NIS side effects, and optimal dose, with or without cyclophosphamide, for patients with recurrent or refractory multiple myeloma. Based on current knowledge of the history of MV infections and the safety of attenuated MV vaccines, the use of MV in an oncolytic context is very promising.

Adenoviruses (Ads) are non-enveloped dsDNA viruses from the Adenoviridae family. Ads have a genome around 38 Kb, which is divided into six regions (E1A, E1B, E2, E3, E4, and L1-5) [141]. These viruses cause upper respiratory tract, gastrointestinal, and ocular infections by entering cells via receptor-mediated endocytosis. The primary receptor involved is the coxsackie-adenovirus receptor (CAR), a membrane glycoprotein in the immunoglobulin superfamily, which is expressed at the cell surface [142]. Interaction of the COOH-terminal knob of the viral fiber protein with the CAR is the first step in adenovirus entry into the host cell. Sequences of the fiber knob amino acid vary among different serotypes of adenoviruses, but almost all serotypes recognize CAR. Simultaneously with receptor recognition and binding by the viral fiber protein, the penton base protein interacts with host cell integrins, which are cell surface adhesion molecules [142]. The penton base-integrin interaction triggers a variety of signals, resulting in cytoskeleton rearrangement and enhanced Ad internalization, via clathrin-coated vesicles, into endosomes. Acidification of the endosome causes penetration of the Ad core into the cytoplasm.

Once the Ad core is in the cytoplasm, disassembly of capsid proteins and uncoating of DNA are necessary for Ad entry into the nucleus [142-144]. In the nucleus, the viral terminal protein associates with the nuclear matrix. Replication of Ad occurs in two phases: the early and late transcription phases. The immediate-early E1A gene is transcribed prior to replication of Ad DNA, and its products regulate transcription of host and viral genes. Transcription of the early genes, E1B, E2A, E2B, E3, and E4, follows E1A transcription. Late phase transcription is initiated as DNA replication begins. Conformational changes in viral chromatin, the viral gene, Iva2, and cellular transcription factors contribute to the transcription of the late genes, which primarily produce structural components of the virion and proteins needed in protein processing and assembly. The virion is assembled within the nucleus, which involves encapsidation of the viral chromosome into a pre-formed empty capsid. No specific pathway for Ad release is currently known; prolonged infection eventually leads to cell lysis [141, 145].

Adenovirus has been studied since the 1950s, and its biology is fairly well known. Ads have several characteristics that make them suitable for use as oncolytic viruses. Their DNA does not integrate into the host genome, they can be produced in high titers, and they have broad tropism, due to the wide expression of their receptors [141]. Ad genomes can be easily manipulated, and various

modifications have been introduced to restrict replication to cancer cells, such as deletion of the E1B gene. The 55 KDa E1B protein is non-essential for viral replication, and is one of the Ad proteins which account for virulence. E1B protein triggers suppression and ubiquitin-mediated degradation of p53 [145-147]. In normal cells, p53 activation of p21 inhibits Ad-induced activation of the transcription of cyclin E, cyclin A, and other cell cycle progression genes. The E1B protein forms a complex with p53 that leads to the degradation of p53, allowing Ad replication in normal cells. Deletion of the E1B gene makes Ads susceptible to the p53 antiviral pathway, but the virus is still able to replicate in p53-defective cells, which include nearly 50% of human tumors [147]. The first engineered Ad with E1B-55K deletion used as oncolytic virus was dl1520 (also called ONYX-015) [148]. The dll520 strain was engineered using the E1A gene from the Ad subtype 2 (Ad2) and the rest of the genome from subtype Ad5. More than fifteen clinical trials have been performed using dll520, with excellent results [145, 149-154]. dll520 has undergone Phase I to III clinical trials, which identified weaknesses, as well as advantages of this virus.

An alternative approach to promoting specificity of Ad replication within tumors is mutation of the E1A gene [155-156]. The E1A protein stimulates S-phase entry, a step needed for transcriptional activation of other early phase viral genes. Ads, such as the Δ24 virus, have lost the capacity to efficiently replicate in normal cells, due to mutations in the E1A gene. Many cancer cells have dysregulated cell

cycles, allowing such viruses to replicate. Success of virotherapy studies with Ads led to the release of these viruses as a standard cancer treatment in China, the first country to permit clinical virotherapy [157].

Herpes simplex virus (HSV) is a large enveloped dsDNA virus belonging to the Herpesviridae family. HSV has a genome of 152 Kb, which encodes over 80 genes [158]. HSV is a know pathogen in humans. HSV-1 has primary sites of replication at the skin, cornea, and mucosa, and HSV-2 at the genital mucosal surfaces. HSV can cause a latent infection, which can last the lifetime of the host. The latent infection can develop into a lytic infection, which can ultimately damage the central nervous system (CNS) or cause meningitis, encephalitis, or genital herpes lesions.

HSV enters the host cell by attachment of viral glycoprotein C to heparan sulfate moieties present on the host cell surface [159]. Viral glycoprotein D is essential for entry and attaches to the cell surface receptor nectin-1. Herpesvirus entry mediator, HVEM, a receptor in the tumor necrosis factor receptor (TNF-R) family, can also attach to HSV glycoprotein D and mediate cell entry. Although various receptors that bind to the viral glycoprotein D have been described, they bind independently and are not co-receptors [158, 160]. Following attachment to the cell surface, the viral envelope and the host cell plasma membrane fuse a process that is dependent on pH and temperature. The viral core is then released

into the cytoplasm and reaches the nuclear pores, through which the viral DNA enters the nucleus.

Transcription and translation of HSV genes occur in a specific order and genes can be divided in three categories: immediate early, early, and late. Immediate early proteins ICP4, ICP27, and ICP0 serve as transcription initiators for early genes. Proteins coded by the early genes downregulate immediate early genes and upregulate late genes [158, 160]. After DNA replication, a capsid containing the viral DNA is formed in the nucleus [158, 161]. The capsid buds through the nucleus membrane and leaves the cell through the Golgi complex. The tegument and envelop are acquired during this process. The ability to establish a latent infection is an important characteristic of HSV [158, 160]. A set of latency-associated transcripts are expressed during latency, which are responsible for neuronal survival during infection, for establishment and maintenance of viral latency, and for reactivation of the virus from latency.

HSV selectivity towards tumor cells was achieved by the introduction of genetic modifications. The first variation of HSV for cancer therapy (dlsptk) was a deletion in the thymidine kinase (TK) gene [162]. This modification allowed the virus to replicate selectively in malignant human glioma xenografts in mice, while sparing normal cells. Although the dlsptk strain was successful, it did not afford the option of HSV treatments using anti-herpetic drugs that targeted the TK gene

[6, 70]. Two other modifications did enable HSV tumor selectivity without interference with the anti-herpetic drug target: the deletion of the main neurovirulence gene, γ34.5, and the inactivation of the viral gene, ICP6, in the G207 strain [70, 163]. Deletion of the γ34.5 gene stopped the ability of HSV to replicate in the CNS. Inactivation of ICP6 inhibited coding of viral ribonucleotide reductase (RR), so that viral DNA replication became dependent on host cell conditions. As dividing cells express the RR protein more than normal cells, replication of G207 is more likely to occur in tumor cells. The oncolytic capacity of HSV has been demonstrated by a number of studies. For example, human renal carcinomas were shown to be susceptible to HSV, as well as prostate tumors *in vitro* and *in vivo* [164-165]. Phase I clinical trials assessing HSV safety demonstrated that the constructions, OncoVEXGM-CSF and NV1020, are well tolerated and can be safely administered to patients with melanoma, breast, neck and head, and gastrointestinal cancers, as well as hepatic colorectal metastases [166-167].

Vaccinia virus (VV) is a large, enveloped, dsDNA virus. VV is the prototype member of the *Poxiviridae* family [168-169]. It is mostly non-pathogenic and was used to develop the vaccine smallpox, which is caused by the variola virus. VV has a 189 Kb genome that encodes more than 250 proteins, including the specific enzymes and factors needed for transcription and replication in the cytoplasm. VV infection and replication starts with the entry of virus particles into the host cell;

entry and binding to the host cell receptor vary according to the type of infectious particle [168-169]. Two infectious forms of VV have been described: the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). The forms differ in surface glycoproteins, the number of wrapping membranes, and host cell receptors. VV receptors are still not known, although glycosaminoglycans and components of the extracellular matrix have been described as VV receptors [170]. Following attachment to the host cell surface, fusion is activated, and the viral and host cell membranes fuse. Entry of IMV and EEV into the cell is likely to differ, because these forms have different numbers of wrapping membranes. A number of entry mechanisms have been proposed, including IMV entry by endocytosis, IMV binding to the host cell surface and fusion with the plasma membrane, extracellular uncoating of IMV and core crossing through the plasma membrane, EEV binding and fusion to the plasma membrane, and EEV endocytosis followed by destruction of the EEV outer membrane by acidified vesicles [168]. Regardless of the form of ingress, entry of both VV forms results in the delivery of the DNA-containing core into the cytoplasm. The virion-associated transcriptional machinery is activated immediately after core entry [171]. The viral RNA polymerase and the early transcription factor (ETF) are responsible for initiation of early mRNA synthesis. VV early genes include encoding factors required for DNA replication, such as DNA polymerase and genes encoding factors. Intermediate genes and viral DNA synthesis are involved in late gene expression, which accounts for transcription of

the majority of the genome. After assembly of the virion, VV exits the infected cell [172]. IMV particles are mainly released following cell lysis. EEV particles are formed by membranes derived from the Golgi network or early endosome wrapping IMV, followed by fusion of the outer membrane with the plasma membrane, exocytosis, and release.

VV is a promising oncolytic virus. It has a short life cycle with a rapid cell-to-cell spread. It has strong lytic capability, and its molecular biology is well known. Research on its use as a vaccine against smallpox has provided knowledge of the responses, adverse reactions and safety profile of this virus in humans. VV infection is not considered a serious health issue, but may cause rash, fever, and head and body aches. Complications can arise in immunocompromised people, such as necrosis at the vaccination site or neurological problems, which can have serious consequences [173]. Similar to other oncolytic viruses, VV has a natural ability to replicate within and lyse cancer cells. Tumor selectivity is possible and related to an activated epidermal growth factor receptor (EGFR)-RAS pathway, which is present in most human cancers. High levels of EGFR in tumor cells activate the RASs pathway, which blocks the activation of antiviral responses and allows VV to replicate.

VV has been extensively used in clinical trials against cancer. Wild-type VV administered via intratumoral injection produced significant antitumor responses

in malignant melanoma and bladder cancer [173]. Four Phase I/II clinical trials showed tumor regression rates greater than 50% (25 of 44 patients) at the site of injection, and complete regression in 25% of patients (11 of 44). Other clinical trials used JX-594, a modified strain of VV, in which the human granulocyte macrophage colony-stimulator factor (GM-CSF) is inserted into the viral thymidine kinase (TK) gene [174-175]. The inactivation of the TK gene increases the tumor tropism of the virus by making it dependent on cellular TK. Cancer cells with cell cycle abnormalities have high concentrations of TK. In one clinical trial using JX-594, three out of ten patients with liver cancer had objective responses, and six had stabilization of the disease. Another trial demonstrated anti-tumor activity in patients with hepatocellular carcinoma. These studies found expression of GM-CSF and increases in neutrophils, eosinophils, and monocytes, suggesting anti-tumor immune responses. Phase I/II and Phase II clinical trials are ongoing to determine the anti-tumor activity and safety of the JX-594 strain (http://clinicaltrials.gov IDs:NCT00429312, NCT00625456, NCT00554372 and NCT01169584).

Vesicular stomatitis virus (VSV) is an arthropod-borne virus, the prototype virus of the Rhabdoviridae family. VSV infects farm animals, such as cattle, horse, and swine; invertebrates and some plants; however, human infection is rare [176]. VSV causes vesicular stomatitis, a disease characterized by vesicular and erosive lesions on the dorsal surface of the tongue, accompanied by vesicles on the gums

and lips [15]. The two major serotypes of VSV on the North American continent are the New Jersey (VSV-NJ) and the Indiana (VSV-Ind) strains, which are distinguished by the antigenic structures of the G protein and the neutralizing antibodies against the G protein [177-178]. Pathogenic outbreaks caused by New Jersey strains are more frequent and more severe than those caused by Indiana strains. Infected arthropods are closely associated with new animal infections, and arthropod biting is believed to be the source of VSV infection. Black flies (*Simulium vittatum*) and sand flies (*Lutzomyia spp*) appear to be part of the transmission cycle, acting as bridge vectors between unknown reservoir systems and domestic livestock, or as maintenance reservoirs through vertical transmission from infected females to offspring [15, 179-180].

VSV is an enveloped, negative-stranded RNA virus with an 11.2 Kb genome encoding five monocistronic mRNAs: nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), and large protein (L) [14, 176, 181]. The G protein is an externally oriented, trimeric, membrane-spanning protein, which is responsible for binding to the host cell surface receptors. Phosphatidylserine (PS) was long thought to be the cell surface receptor for VSV. PS is a phospholipid component of cell membranes widely distributed among animals, plants, and microorganisms [182-183]. PS plays an important role in cell-to-cell communication, as well as transfer of biochemical messages into the cell which trigger cellular responses. Although PS turned out not to be the VSV receptor,

binding to PS is probably important in a subsequent step of the entry process [184-186]. VSV binds to cells via an unknown receptor and fuses, in a pH-dependent manner, with endosomal membranes [14-15, 187]. G protein produced by VSV catalyzes the fusion of viral and cellular membranes, releasing the viral ribonucleoprotein (RNP) into the cytoplasm. A decrease in the endosomal pH induces membrane fusion and releases the viral core into the cytoplasm of the host cell.

Once the viral core is released, VSV must replicate within the cell in order to survive. To produce a positive-sense RNA template for replication, VSV carries its own polymerase. Together, L, N, and P proteins serve as the viral transcriptase, responsible for transcription and replication of the genome [70, 181]. Polymerase transcribes a leader RNA beginning at the 3' end of the genome, and sequentially synthesizes the five monocistronic mRNAs, which are capped and methylated at their 5' terminus, and polyadenylated at their 3' terminus [176, 181, 188-189]. Viral polymerase produces the positive-strand RNA from which further copies of the negative-strand genome are prepared and packaged as viral progeny [14-15, 181, 188]. VSV assembly is largely carried out by the M protein, which condenses the viral nucleocapsid, causing its collapse into the characteristic tightly coiled, bullet-like shape [190]. Translation of VSV mRNAs is highly dependent on the host cell translation machinery. To increase viral protein synthesis, VSV modifies the host cell machinery to decrease synthesis of host cell

proteins [190]. The nuclear pore complex (NPC) is a macromolecular complex within the host, whose components mediate the exchange of molecules between the nucleus and the cytoplasm [191-192]. The NPC is regulated by signaling pathways, and by viruses which have developed mechanisms to interfere with nucleocytoplasmic trafficking. The relatively small matrix (M) protein of VSV is responsible for shutting off export of host-cell mRNA from the nucleus [16-17]. Interaction of M protein with Nup98 and Rae1 leads to disruption of major host cell functions in an attempt to prevent antiviral gene expression.

Table 1. Cancer-selectivity mechanisms of oncolytic viruses

Virus	Class Family	Characterisitccs	Human pathogen	Selectivity	Stage of Development
Newcastle Disease virus (NDV)	ssRNA Paramyxoviridae	Naturally occurring virus with oncolytic capacity. NDV enters the cell through attachment of hemagglutinin-neuraminidase (HN) to sialic acid receptors	No	Cells with defective IFN production or resistant to IFN treatment PKR deficient cells	Phase I/II
Vesicular Stomatitis Virus (VSV)	ssRNA-, Rhabdovirridae	Live attenuated and recombinant derivatives have being used as oncolytic viruses. Naturally occurring strains with mutated Matrix (M) protein are more effective as oncolytic agents.	No	Profit from an impaired IFN pathway Constitutive activation of the RAS/Raf1/MEK pathway	Pre-clinical
Adenovirus (Ad)	dsDNA Adenoviridae	The primary receptor involved is the CAR receptor Ad genomes can be easily manipulated and various modifications have been introduced to restrict replication to cancer cells	Yes	p53 null/mutant or/and inactivated p53 pathway	Phase III
Reovirus	dsRNA, Reoviridae	Naturally occurring virus with oncolytic capacity Reovirus enters the host by either respiratory or enteric routes and infects the epithelium and associated lymphoid tissue	Yes	Ras-activated pathway PKR deficient cells	Phase I/II
Herpes Simplex Virus (HSV)	dsRNA Herpesviridae	HSV enters the host cell by attachment of viral glycoprotein C to heparan sulfate moieties present on the host cell surface Modifications in the TK allow for selective replication in tumor cells	Yes	Ras-activated pathway Infects highly replicating cells	Phae I/II
Measles virus (MV)	ssRNA Paramyxoviridae	Oncolytic MV vaccines consist on live-attenuated or engineered virus. Infects host though the respiratory tract Cell penetration occurs via virus-cell membrane fusion mediated by CD46 and SLAM receptors	Yes	Cells with abundant expression of CD46 Cells with defects in the IFN response. PKR deficient cells.	Phase I/II
Vaccinia virus (VV)	dsDNA Poxiviridae	VV has a short life cycle with a rapid cell-to-cell spread. It has strong lytic capability, and its molecular biology is well known	Yes	Activated epidermal growth factor receptor (EGFR)-RAS pathway, present in most human cancers	Phase I/II

HOST ANTIVIRAL RESPONSE TO VESICULAR STOMATITIS VIRUS

Host Response Machinery

The first line of protection against a pathogen is the innate immune system, which provides immediate defense against infection. The primary functions of the innate immune system are early pathogen recognition and consequent activation of pro-inflammatory responses to the invading pathogens. In contrast to the adaptive immune system, the innate immune response is not antigen-specific and reacts in the same way to a variety of organisms [193-194]. The adaptive immune system responds specifically to antigens, and its response might take days to develop. Adaptive response relies primarily on receptors on the surface of B and T lymphocytes, antibody production and activation of macrophages and NK cells for pathogen elimination. Adaptive response also involves immunological memory, a process that maintains and enhances future responses to a given antigen throughout the life of the host.

The first host defense against potential pathogen infection are anatomical barriers [193-194]. Epithelial surfaces, like the skin, are an impermeable barrier to some infectious agents. Ciliary movement and peristalsis remove microorganisms from respiratory and gastrointestinal passages. Secretion of toxic substances by the normal flora of the gastrointestinal tract can prevent colonization of pathogenic microorganisms.

Once infectious microorganisms penetrate the physical barriers, the innate immune system relies on a variety of hematopoietic cells – macrophages, natural killer (NK) cells, NK T cells, dendritic cells, eosinophils, neutrophils, mast cells, and basophils – as well as skin and endothelial cells lining the respiratory, gastrointestinal, and genitourinary tracts, to respond to an infection [193, 195]. Monocytes circulate in the peripheral bloodstream, then enter tissues and differentiate into macrophages, which are activated upon infection. Once activated, macrophages engulf the microorganisms, a process known as phagocytosis, and digest the foreign elements. Proteolysis by macrophages produces peptide fragments in forms that can activate T-cell responses.

NK cells, which are ready to kill as soon as they are formed, attach to pathogens and release enzymes and other substances that damage their outer membranes [193-194]. NK cells are able to target and kill not only infected cells, but also altered or cancerous cells. Molecules present at the NK cell surface are responsible for recognition of the target cell, ultimately inducting apoptosis in the affected cells. NK cells are also important immunomodulators, producing and secreting cytokines that regulate some functions of T and B lymphocytes. NK T cells, which share characteristics of both NK cells and T cells, are a distinct lineage of $CD3^+$ T cells that exhibit NK surface antigens. NK T cells represent a link between innate and adaptive immune responses. They secret large amounts of

cytokines and provide a faster cell-mediated immune response than conventional T-helper cells, which require several days.

Dendritic cells are professional antigen-presenting cells found in the skin, lymph nodes, and tissues throughout the body [193, 195]. Like macrophages, dendritic cells are phagocytes derived from monocytes; their primary function is to ingest antigens and break them into fragments. Once dendritic cells take up the antigens, they become activated by cytokines and enter the lymphoid organs. They then present the antigen fragments to naïve T4-lymphocytes via MHC-II molecules, or to naïve T8-lymphocytes via MHC-I molecules, which become activated, proliferate, and differentiate into effector cells.

Eosinophils are found in the bloodstream and are also capable of ingesting microbes. However, they are more effective against foreign bodies that are too large to be phagocytized, such as helminths and other parasites [193-194]. Eosinophils contain cytoplasmic granules containing toxic substances and enzymes that are released when targeted cells are encountered. The substances and enzymes make holes in the target cell's membrane.

Neutrophils are the most common white cell in the bloodstream, and are among the first cells to respond to infection [193]. Neutrophils migrate from blood vessels to the affected tissue, where they produce large quantities of reactive oxygen species that are toxic to certain pathogens. Neutrophils also have phagocytic capacity, sequestering microbes and processing antigens that are

internally degraded. Neutrophils also produce cytokines and act as immunomodulators.

Mast cells and basophils are morphologically similar and act as first responders to infection [193]. These cells do not ingest microbes, but respond to helminthic parasites and allergic reactions. They contain histamine-filled granules, which when released, increase blood flow and stimulate inflammation in the damaged tissue.

Pathogen recognition

An important feature of the innate immune response is the recognition of microbial-associated molecular patterns (MAMPs), conserved microbial components that are shared by a large number of pathogens, and non-pathogenic microorganisms [196-197]. DAMPs are damage (or danger)-associated molecular patterns that are released from disrupted or injured cells and trigger immune responses and activating repair mechanisms through their interaction with pattern recognition receptors (PRRs). In tumor cells infected with an oncolytic virus, release of danger signals can trigger an antitumor immunity by recruitment of cells of the innate immune system into the tumor tissue [121, 198-199]. Conversely, viral infection can lead to cell lysis which can trigger an autoimmune response by release of danger signals from inside the infected cell resulting in an hyperactive immune system attacking normal tissues as if they were foreign

organisms. For instance, IFNα induced by the enterovirus Coxsackievirus B can have a deleterious function, acting as an initiator of the autoimmunity directed against β cells which causes type 1 diabetes mellitus [200]. PRRs are proteins expressed in cells of the innate immune system, which are responsible for recognition of MAMPs and DAMPs and initiate the host's innate immune defenses. PRRs fall into multiple categories (Table 2). The first group contains secreted molecules that circulate in blood and lymph, such as C-reactive protein and peptidoglycan recognition proteins (PGRs) [197, 201]. These molecules scrutinize blood and lymph, and bind to MAMPs once they are detected. This interaction triggers the complement cascade, leading to the opsonization of the pathogen, which facilitates phagocytosis. The second group of PRRs is the surface receptors on phagocytes, such as macrophages and dendritic cells, which bind the pathogen for engulfment [201-202]. Mannose, scavenger, and opsonin receptors are examples. These receptors activate classical and lectin pathways of the complement system, and opsonize pathogens for phagocytosis. The third group of PRRs are the cell-surface receptors, which senses the pathogen, initiating a signal leading to the release of effector molecules [197, 201-202]. This group contains a set of transmembrane receptors, the toll-like receptors (TLRs) that recognize different types of MAMPs (Table 2; Figure 1). The function of various TLRs depends on their localization [202]. Eleven type I transmembrane proteins have been identified as TLRs (TLR1-TLR11). These receptors contain a leucine-rich ectodomain that mediates the recognition of MAMPs, a transmembrane

domain, and an intracellular Toll-interleukin 1 receptor (TIR), which is necessary for downstream signal transduction. TLRs 1, 2, 4, 5, 6, and 11 are localized at the plasma membrane, while TLRs 3, 7, 8, 9, and 10 are in the endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes (Table 2; Figure 1) [197, 201-202]. TLR1 forms a heterodimer with TLR2, which recognizes triacylated lipopeptides from Gram-negative bacteria and mycoplasma. TLR2 distinguishes a wide range of PAMPs derived from bacteria, fungi, parasites and viruses. TLR3 identifies poly(I:C) – a synthetic analog of double-stranded RNA (dsRNA), genomic RNA of reoviruses, and dsRNA produced during the replication of single-stranded RNA (ssRNA), viruses, and certain small interfering RNAs. TLR4 responds to bacterial lipopolysaccharide (LPS). TLR5 recognizes flagellin, a protein from bacterial flagella. TLR6 form a heterodimer with TLR2, which recognizes diacylated lipopeptides from Gram-positive bacteria and mycoplasma. TLR7 recognizes imidazoquinoline derivatives, such as imiquimod and resiquimod (R-848), and guanine analogs. TLR7 also recognizes ssRNA derived from RNA viruses, synthetic poly(U) RNA, and certain small interfering RNAs. Human TLR8 mediates the recognition of R-848 and viral ssRNA. TLR9 recognizes unmethylated 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA motifs that are often present in bacteria and viruses. TLR10, which is non-functional in mice, appears to be functional in humans, but no ligand has been identified. Finally, TLR11 recognizes uropathogenic bacterial components and

profilin-like molecules derived from *Toxoplasma gondii* (Table 2; Figure 1) [202].

Many viruses perform their entire replication cycle in the cytoplasm, while others navigate through the cytoplasm on their way out of the cell. Host cells have developed receptors that identify these viruses. These receptors are the fourth group of PRRs and the most recent receptors to be identified: the cytosolic nucleotide sensors, which sense cytoplasmic DNA and RNA (Table 2; Figure 1) [203-204]. Retinoic acid–inducible gene I (RIG-I) was identified in 2004 as a cytosolic sensor of dsRNA. Melanoma differentiation-associated gene 5 (MDA-5) was identified soon after as a CARD-containing helicase that detects viral RNA. Together with the laboratory of genetics and physiology 2 (LGP-2), these cytoplasmic sensors form the RIG-I-like receptor (RLR) family. Cytoplasmic RLRs are specialized in recognition of viral elements that are exposed during their cytosolic replication [203, 205]. MDA-5 efficiently recognizes the dsRNA mimetic poly(I:C), while RIG-I identifies 5'-triphosphorylated single-stranded RNA from both positive- and negative-stranded viruses. The three receptors have an internal DExD/Hbox RNA helicase domain, which is activated by ligand binding. RIG-I and MDA-5 signal through their N-terminal caspase activation and recruitment domains (CARDs). In contrast to RIG-I and MDA5, which induce type I IFN, LGP-2 lacks CARDs and blocks IFNα/β production. Another two groups of cytosolic MAMPs and DAMPs sensors are the cytosolic nucleotide-

binding and oligomerization domain-like receptors (NLRs) and the IFI200 family member absent in melanoma 2 (AIM2) (Table 2) [206]. NLRs are a receptor family characterized by the presence of a conserved NOD motif. NLR receptors are significant in initiating an inflammatory reaction, which in turn triggers cascades of innate and adaptive immune responses. AIM2 is an IFN-I–inducible cytosolic protein containing pyrin (PYD) and hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats (HIN200) domains [207]. Among other functions (such as cell proliferation), AIM2 was shown to be a cytoplasmic sensor of DNA through interaction with the apoptosis-associated speck-like protein (ASC) through its PYRIN domain. Sensing of cytoplasmic DNA triggers formation of the AIM2 inflammasome which activates caspase-1 and the ASC pyroptosome, which induces pyroptotic cell death in cells containing caspase-1 [208-209]. Inflammasomes are intracellular multiprotein complex which leads to the cleavage of procaspase-1 to active caspase-1 that in turn activates interleukins (IL)-1β and -18. IL-1β and IL-18 are pro-inflammatory cytokines that regulate immune and inflammatory responses (Table 2; Figure 1) [210]. In addition to AIM2, NLR family, pyrin domain containing 1 (NLRP1 or NALP1), NLR family, pyrin domain containing 3 (NLRP3, NALP3 or cryopyrin) and NLR family, caspase recruitment domain (CARD) containing 4 (NLRC4) are also part of the inflammasome complex. In the same way than AIM2, these proteins mediate the assembly of the inflammasome through NOD-mediated oligomerization and interaction with caspase-1 via the adaptor ASC. NLRP1 senses bacterial muramyl

dipeptide (human) and *Bacillus anthracis* lethal toxin in mice. NLRC4 senses several Gram-negative bacteria (i.e *Legionella pneumophila*, *Pseudomonas aeruginosa*) while NLRP3 senses viral infection (i.e influenza A virus, encephalomyocarditis virus – EMCV, vaccinia virus Ankara strain and adenovirus,) [206]. Recently, it has been demonstrated that the cytosolic sensor of RNA viruses RIG-I is also able to engage the caspase-1 pathway, through interaction with the ASC adaptor protein and inflammasome formation, which leads to release of the pro-inflammatory cytokines IL-1β and IL-18 thus recruiting immune cells to the site of infection (Table 2; Figure 1) [211]. Caspase-1 is a cysteine protease essential for the inflammasome's proteolytic function. Once recruited to an inflammasome, proteolytic processing of caspase-1 occurs which generates two subunits (10 (p10) and 20 (p20) kilodalton). The activated caspase-1 converts proIL-1β into its active 17 kilodalton form; proIL-18 is also converted into its 18 kilodalton active form by caspase-1 [210]. Activation of IL-1β engages mononuclear and endothelial cells which are critical effectors in the host response to pathogens. IL-18 stimulates interferon-γ production and type 1 helper T cells (Th1) differentiation which activates macrophages and proliferation of cytotoxic $CD8^+$ T cells.

Figure 1. Components of the TLR and IFN signaling pathways and intracellular pattern recognition receptors.

MyD88 is the key signaling adaptor for all TLRs (with the exception of TLR3 and certain TLR4 signals). Its central role is the activation of NF-κB. It is directly recruited to the TIR domains in certain TLRs and recruits IRAK4. This triggers activation of a pathway involving IRAK1, TRAF6 and TAK1. In the signaling by TLR7, TLR8 and TLR9, the MyD88–IRAK4 pathway also leads through TRAF6 and TRAF3 to the activation of IRF7. Also MyD88/IRAKs/TRAF6 leads to the activation of the IKK, which consists of IKK-α, IKKβ- and NEMO. This pathway is used by TLR1, TLR2, TLR4, TLR5 and TLR6 in the plasma membrane and releases NF-B from its inhibitor so that it translocates to the nucleus and induces expression of inflammatory cytokines. Activation of IRF-3 and IRF-7 by TLR3 and TLR4 is MyD88-independent. The TRIF adaptor is essential for the MyD88-independent pathway. The IKK-ε and TBK1 kinases mediate activation of IRF3 and IRF-7 downstream of TRIF. Cytosolic dsRNA or 5'-triphosphate ssRNA is recognized primarily by the cytoplasmic RNA helicases RIG-I and MDA5, which mediate interaction with the adaptor IPS-1, localized to mitochondria, and trigger signaling to NF-κB and IRF3. ssRNA is recognized by TLR7/8 and dsRNA is recognized by TLR while TLR9 recognizes CpG-containing DNA in endosomes and induces signaling to IRF7 as well as to NF-κB. Inflammasomes form large multimolecular complexes that control the activity of caspase-1. Activation of the NLRP3 inflammasome assembly results in activation of caspase-1, which proteolytically activates IL-1β and IL-18 cytokines. Caspase-1 is recruited into the complex via the adaptor protein apoptosis speck protein with CARD (ASC). Caspases are activated through autoproteolytic cleavage and can then process their substrates. The produced pro-inflammatory IL-1β family cytokines can act on other cell types or act in a feed-forward loop through its respective receptors.

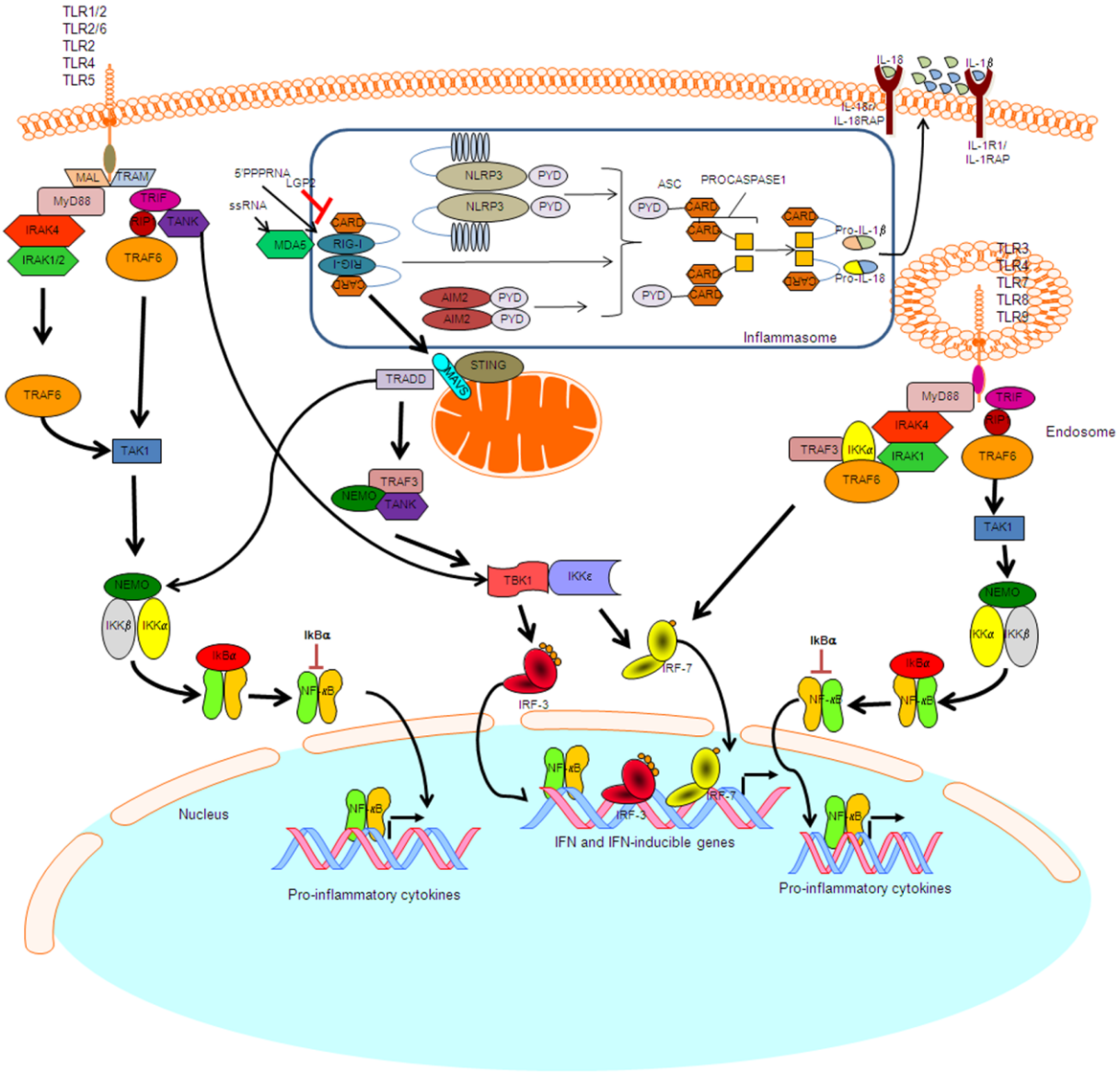

TLR1/2
TLR2/6
TLR2
TLR4
TLR5
IL-18
IL-1β
IL-18α/
IL-18RAP
IL-1R1/
IL-1RAP
MAL
TRAM
MyD88
TRIF
RIP
TANK
IRAK4
IRAK1/2
TRAF6
5'PPPRNA
LGP2
ssRNA
MDA5
RIG-I
CARD
NLRP3
PYD
ASC
PROCASPASE1
Pro-IL-1β
Pro-IL-18
AIM2
Inflammasome
TLR3
TLR4
TLR7
TLR8
TLR9
Endosome
TRADD
STING
TAK1
TRAF3
NEMO
TANK
TBK1
IKKε
IKKβ
IKKα
IkBα
NF-κB
IRF-3
IRF-7
Nucleus
Pro-inflammatory cytokines
IFN and IFN-inducible genes

Table 2. Microbial-associated pattern recognition (MAMPs) by pattern-recognition receptors (PRRs)

Receptor	Specie	Localization	Expression (Cell Type)	Ligand	Origin (of Ligand)	Adaptor Protein	Transcriptional Factor	Induced Cytokines
Toll Like Receptors (TLRs)								
TLR1/2	Human Mice	Plasma Membrane	Ubiquitous	Triacyl lipopetides Soluble factors	Bacteria and Mycobacteria *ningitidis*	MyD88	NF-κB	Inflammatory cytokines (i.e TNFα)
TLR2	Human Mice	Plasma Membrane	DCs, PMLs and monocytes	Heat Shock protein 70 Peptidoglycan Lipoprotein/lipopeptides	Host Gram-positive bacteria Various pathogens	MyD88	NF-κB	Inflammatory cytokines (i.e TNFα
TLR3	Human Mice	Endosome	DC and NK cells Upregulated on epithelial an endothelial cells	Double-stranded RNA	Viruses	TRIF	NF-κB IRF-3 IRF-7	Inflammatory cytokines (i.e TNFα) Type I IFN
TLR4	Human Mice	Plasma Membrane	Macrophages PMLs, DCs, ECs	Lipopolysaccharides Envelope protein Taxol	Gram-negative bacteria Mouse mammary-tumor virus Plants	MyD88 TRAM TRIF	NF-κB IRF-3 IRF-7	Inflammatory cytokines (i.e TNFα) Type I IFN
TLR5	Human Mice	Plasma Membrane	Monocytes Immature DCs Epithelial cells NK cells T cells	Flagellin	Bacteria	MyD88	NF-κB	Inflammatory cytokines (i.e TNFα and IL-6)
TLR6/2	Human Mice	Plasma Membrane	B cells	Zymosan Lipoteichoic acid Diacyl lipopetides	Fungi Gram-positive bacteria Mycoplasma	MyD88	NF-κB	Inflammatory cytokines (i.e TNFα)
TLR7	Human Mice	Endosome	B cells pDCs	Single-stranded RNA (ssRNA) Imidazoquinoline	Viruses Synthetic compounds	MyD88	NF-κB IRF-7	Inflammatory cytokines (i.e TNFα) Type I IFN
TLR8	Human Mice	Endosome	Monocytes	Single-stranded RNA (ssRNA) Imidazoquinoline	Viruses Synthetic compounds	MyD88	NF-κB IRF-7	Inflammatory cytokines (i.e TNFα) Type I IFN
TLR9	Human Mice	Endosome	pDCs B cells Macrophages PMLs NK cells Microglia cells	CpG-containing DNA	Bacteria, Malaria and Viruses	MyD88	NF-κB IRF-7	Inflammatory cytokines (i.e TNFα) Type I IFN
TLR10	Human Mice (analog to TLR10)	Plasma Membrane	B cells pDCs	Not determined	Not Determined	MyD88	NF-κB	Inflammatory cytokines
TLR11	Mice	Plasma Membrane	Not determined	Profilin-like molecule	*Toxoplasma gondii*	MyD88	NF-κB	Inflammatory cytokines (i.e TNFα) Type I IFN
Cytosolic receptors								
RIG-I	Human	Cytoplasm	Ubiquitous	Short dsRNA 5' triphosphate RNA	RNA viruses DNA viruses	MAVS	NF-κB IRF-7	Type I IFN IL-1β IL-18
MDA5	Human	Cytoplasm	Ubiquitous	Long dsRNA	RNA viruses	MAVS	NF-κB IRF-7	Inflammatory cytokines (i.e TNFα) Type I IFN
NLRP1	Human Mice	Cytoplasm	DCs B cells Macrophages	Peptidoglycan Letal toxin (LT)	Bac *Bacillus anthracis* teria	ASC Caspase 1		IL-1β
NLRP3	Human Mice	Cytoplasm	pDCs B cells Macrophages	LPS, RNA, toxins RNA Letal toxin (LT) Poly I:C Imidazoquinoline Amyloid-β particles	Bacteria Viruses *Bacillus anthracis* Synthetic compounds Host	ASC Caspase 1		IL-1β IL-18
NLRC4	Human Mice	Cytoplasm	pDCs B cells Macrophages	Flagellin	Bacteria	ASC Caspase 1		IL-1β IL-18
AIM2	Human	Cytoplasm	pDCs B cells Macrophages	Cytosolic DNA	DNA viruses Intracellular bacteria	ASC Caspase 1		IL-1β IL-18

Host recognition and defense against VSV

Host recognition of VSV infection involves RIG-I and TLR7, and stimulates production of type I IFNs [212-213]. RIG-I is a soluble protein found in the cytosol of many cell types, and it plays a major role in recognition of VSV in cDCs, macrophages, and fibroblasts [205]. RIG-I contains a DExD/H box RNA helicase and two caspase recruiting domain (CARD)-like domains. The helicase domain interacts with dsRNA, and the CARD domains are required to transmit the signal. Upon sensing VSV, RIG-I signals through the mitochondrial anti-viral signaling (MAVS) adaptor protein via CARD domain interactions. MAVS then associates with the tumor necrosis factor-receptor associated factor 3 (TRAF3), followed by activation of TANK-binding kinase 1 (TBK1) and IkappaB kinase-epsilon (IKK-ε) [214]. A recently discovered endoplasmic reticulum adaptor facilitates activation of the innate antiviral signaling [215]. Stimulator of interferon genes (STING) activates the nuclear factor-KappaB (NF-κB) and the interferon regulatory factor 3 (IRF-3) transcription pathways, inducing type I IFN. Cells expressing STING are resistant to VSV infection, and loss of STING renders the cells susceptible to VSV infection. STING interacts directly with RIG-I and forms a complex with MAVS.

VSV enters the cell via endocytosis, and TLR7 recognition of VSV infection in the endosome is linked to viral fusion and uncoating. In DCs, TLR-7 senses VSV and signals through a MyD88-dependent pathway [213]. Type I IFN secretion by

pDCs relies significantly on TLR-7/myeloid differentiation factor 88 (MyD88) [216]. The receptor is activated upon engagement of TLR7 and interacts with MyD88, which forms a complex with interleukin-1-receptor (IL-1R)-associated kinase-4 (IRAK-4), IRAK-1, TRAF3, tumor necrosis factor-receptor associated factor 6 (TRAF6), Ikappa B kinase alpha (IKKα), and interferon response factor-7 (IRF7) [205]. The development of this complex activates IRF7 and nuclear factor-kappa B (NF-κB), resulting in production of type I IFNs and cytokines.

Sensing of VSV by either RIG-I or TLR7 culminates in activation of IRF-3/7 and/or NF-κB, factors that are central to the cytokine activation mechanisms leading to antiviral responses. IRF-3 and IRF-7 are members of the interferon regulatory transcription factor (IRF) family [217]. IRF-3 is constitutively expressed in the cytoplasm and remains inactive until activation occurs through phosphorylation of the C-terminal regulatory domain by TBK1 and IKKε kinases [214, 218]. Following phosphorylation, a homodimer formed by dimerization of IRF-3 translocates to the nucleus [219-220]. In the nucleus, the IRF-3 dimer associates with two histone acetyltransferases, cAMP-responsive element-binding protein (CREB) binding protein (CBP) and p300 coactivators. The IRF-3/CBP/p300 complex binds the consensus sites, called IFN-stimulated response elements (ISREs), inducing transcriptional activation of IFN-β. Finally, IRF-3 is targeted for proteasomal degradation [221].

In contrast to IRF-3, IRF-7 is not normally present in most cells, but is expressed at low levels in lymphoid tissues. The low levels of IRF-7 found in unstimulated cells are critical for activating the initial phase of gene induction. Once initial activation of IFN genes is achieved, by IRF-7 (and IRF-3), a second cascade of IFN-α subtypes is triggered and a complete antiviral state is established [222]. Similar to IRF-3, IRF-7 is activated via phosphorylation by TBK1 and IKKε kinases [214]. In pDCs, robust IFN production depends on the MyD88–IRF-7 signaling pathway, in which kinases from the IRAK family phosphorylate IRF-7 [223]. Following phosphorylation, IRF-7 dimerizes and translocates to the nucleus, where it regulates transcription of type I IFN-α genes and several ISGs [224-225].

NF-κB is a transcription factor which exists as inactive dimeric NF-κB proteins, sequestered in the cytosol by IκB proteins. Dimeric DNA binding complexes are formed by interactions among five members of the Rel gene family: RelA (or p65); c-Rel; RelB; NK-κB1, which encodes the p50 subunit and its precursor, p105; and NF-κB2, which encodes p52 and its precursor, p100 [226-227]. Upon signaling through the PRRs, the IκB kinase (IKK) complex is activated, a heterotrimer formed by the kinases, IKKα, IKKβ, and IKKγ/NEMO (NF-κB essential modulator). Once the complex is activated, IKKβ kinase phosphorylates IκB proteins, triggering IκB proteasomal degradation that releases NF-κB dimmers which translocate to the nucleus. In the nucleus, NF-κB is involved in the regulation of genes involved in immune function, including several antiviral

genes. In addition to its role in the immune response, NF-κB is a key regulator of cell survival, cell death, and inflammation.

The type I IFN family includes more than a dozen members with similar functions. IFN-β and IFN-α4 (in mice) and IFN-α1 (in humans) Zcdfza43eare transactivated, following virus-induced activation of IRF-3 and IRF-7. IRF-7 expression is greatly increased by these proteins, transactivating multiple IFN genes [85]. IFN-β is secreted from infected cells and activates type I IFN receptor (IFNAR), which signals through the JAK-STAT pathway. The JAK-STAT pathway involves phosphorylation of the signal transducer and activator of transcription (STAT) proteins by the Janus kinases, tyrosine kinase 1 (JAK-1) and tyrosine kinase 2 (TYK-2). Phosphorylated STAT proteins, STAT-1 and STAT-2, recruit interferon regulatory factor 9 (IRF-9) to form the IFN-stimulated gene factor 3 (ISGF-3) complex. ISGF-3 complex translocates to the nucleus and binds to the IFN-stimulated response element (ISRE) in the promoter region of IFN-stimulated genes (ISGs).

ISGs have been directly implicated in instigation of the antiviral state. Three main type I IFN-induced antiviral pathways have been firmly established: protein kinase R (PKR), the 2-5 OAS/RNaseL system, and the Mx proteins [228]. PKR is produced in an inactive form, and is activated by dsRNA produced during viral replication. PKR phosphorylates translation initiation factor 2a (eIF2a),

preventing recycling of eIF2a, stopping initiation, and inhibiting cellular and viral translation [85, 181]. PKR activation involves a kinase-independent process that also leads to the release of NF-κB from IκB, which sends an anti-apoptotic signal. OAS is also synthesized in an inactive form and is activated by dsRNA produced during viral replication. OAS protein binds to oligomerized ATP. The complex activates RNAseL, which mediates viral and cellular RNA degradation. MX proteins are IFN-induced members of the dynamin superfamily of large GTPase [85, 228]. Mx proteins depend on IFN for their expression, and inhibit virus infection by blocking early stages of the viral replication cycle. Other protein with important antiviral activities is ISG15, one of the most induced ISGs [85, 228]. The ubiquitination response mediated by an ISG, is called ISGylation. More than one hundred ISG15 targets have being identified, many of which are important in the type I IFN response, such as STAT1, RIG-I, MxA, and PKR. Rather than inducing protein degradation, ISG15 prevents IRF-3 degradation, increasing the induction of IFNβ expression. Ubiquitination of IRF-3 can display both positive and negative regulatory functions. Phosphorylation-dependent ubiquitination is well established to terminate IRF3 function by proteasome-dependent degradation. Although IRF-3 degradation mechanism is still unknown, the peptidyl-prolyl cis/trans isomerase (Pin1) protein is known to interact with phosphorylated IRF3 and promotes its ubiquitination. In the positive function, the ubiquitin-like protein ISG15 conjugate with IRF-3, a process mediated by the HERC5 (hect domain and RLD 5) E3 ubiquitin-protein ligase. The interaction

ISG15-HERC5-IRF-3 attenuates the interaction with Pin1 resulting in stabilization of IRF3 [229].

THE ONCOLYTIC VESICULAR STOMATITIS VIRUS

Type I IFNs exhibit potent antiviral activity and are produced by most cells in response to viral infection [230]. VSV is extremely sensitive to the IFN antiviral response [231], and control of VSV replication and spread by IFNs has been well studied [11, 18-20, 232]. Both IFN and IFN-inducible, double-stranded RNA-dependent protein kinase (PKR) play a role in the inhibition of VSV replication [21]. Following infection by VSV, dsRNA is detected by PRRs, which activate a cascade of signaling factors that induce synthesis of type I IFNα/β [205, 230]. In response, a series of ISGs are expressed which inhibit viral replication, including PKR which plays a central antiviral role in cells infected with VSV [22, 205, 233-237].

Type I IFN, secreted following viral infection, induces transcription of PKR kinase [21, 205, 238]. Activation of PKR by viral dsRNA, an early event in the inhibition of viral transcripts, begins with dimerization and autophosphorylation of PKR, which in turn, phosphorylates eukaryotic initiation factor, eIF2, resulting in the inhibition of protein synthesis [181, 205, 237]. Translation control downstream of PKR activation and eIF2 phosphorylation is deregulated in many transformed cells [19]. Therefore, defects in the IFN and PKR systems, which are

present in many transformed and cancer cells, greatly facilitate VSV infection [13, 18-22, 70].

Matrix (M) protein of VSV plays an important role in viral evasion by inhibiting host gene expression [17, 239-240]. Consequently, mutations in the M protein should diminish viral replication, attenuate viral cytotoxicity, and improve oncolytic capacity. Naturally occurring VSV variants have been identified as potent oncolytic viruses with superior therapeutic indices [11]. The AV1 mutant differs from wild-type VSV by a single amino acid substitution (M51R), while AV2 has two mutations (V221F and S226R) in the M protein. AV1 and AV2 strains were first identified as VSV variants that establish persistent infections *in vitro* [241-243]. Later studies showed that these strains fail to suppress IFN production in healthy cells; thus, nonmalignant cells infected by AV1 or AV2 are able to build strong antiviral responses [11]. Further genetic manipulation of VSV has created strains with increased oncolytic capability compared to the wild-type. The deltaM51 virus (ΔM51), in which a fragment of the gene encoding for the M protein is deleted, exhibited high affinity for human high-grade malignant glioma cells, and suppressed their growth as xenograft tumors in nude mice [11]. The engineered rM51R-M strain is a strong candidate as an oncolytic virus for therapies of prostate tumors [25]. These mutants elicit a strong IFN response in healthy cells and restrict VSV replication to cells with defective IFN-signaling; therefore, they offer promise in the development of virotherapy against cancer.

THE USE OF VSV AS ONCOLYTIC VIRUS: OBSTACLES AND ALTERNATIVES

Despite encouraging results from pre-clinical and clinical studies, RNA OV therapy requires further refinement to become established as a cancer-fighting strategy [244-245]. Inefficient viral replication in primary cancers and tumor-specific resistance to OV-mediated killing are two of the obstacles to the application of oncolytic viruses [6, 62-63, 72, 246-247]. VSV is no exception. Although VSV oncolysis is facilitated by defects in the IFN antiviral response, other host factors, including the host immune system and defects in the apoptotic pathway, appear to influence its effectiveness as an OV.

Various strategies have been used to overcome obstacles to the use of VSV as an OV. Histone deacetylase inhibitors (HDIs) complemented VSV virotherapy by facilitating tumor cell infection and killing *in vivo* tumors that had an impaired antiviral response [248]. Neutralization of injected OVs by circulating antibodies in immunocompetent animals was sufficient to ablate delivery of naked virions to tumors [244, 249-250]. To circumvent this problem, cellular carriers were developed to shield the OV from neutralization [39, 251-255]. This strategy holds promise, as cell carriers utilized in the context of murine models have efficiently delivered VSV to tumors in the presence of virus-neutralizing antibody. In mice with B16-ova tumors, naive lymphocytes loaded with VSV purged metastatic

tumors and generated potent T-cell responses, demonstrating that oncolysis in lymphoid organs primes antitumor immunity [256].

Pre-conditioning of immune-competent mice with Treg depletion and IL-2, prior to adoptive T-cell therapy with cells loaded with VSV, significantly enhanced antitumor therapy [257]. IL-2 expressed by infected cells initiated a broad cascade of immunological effects in the microenvironment of the vaccine. When expressed in recombinant VSV, IL-12 successfully reduced tumor volumes and provided substantial survival benefit to animals with head and neck carcinomas [258]. Treatment of a resistant lymphoma with small molecule inhibitors of the antiapoptotic protein BCL-2 enhanced VSV-mediated oncolysis [39]. In primary B-cell chronic lymphocytic leukemia (B-CLL), e*x vivo* B-CLL cells were resistant to VSV oncolysis, partly due to overexpression of BCL-2, which causes a block in the intrinsic mitochondrial pathway. BCL-2 inhibitors restored the mitochondrial apoptotic pathway and synergized with VSV to target and kill the malignant cells without affecting viral replication [39]. The sensitization to VSV oncolysis achieved by these proapoptotic agents identifies a potential therapeutic combination for apoptosis-resistant malignancies. Several BCL-2 family member inhibitors are currently in advanced pre-clinical and clinical trials, as single agents or in combination with conventional chemotherapy [46, 259-260]. However, further studies are needed to increase the range of cancers that could benefit from this type of combination therapy. OV therapy in combination with chemotherapy

drugs may become more popular in future clinical trials. Experimental pre-clinical studies have shown that the combination of VSV OV with cytotoxic or immunotherapic agents can be more efficacious for cancer therapy than VSV OV alone. Overall, combination therapy for tumors using VSV is a promising clinical strategy, but further investigation of effectiveness, safety, and feasibility is necessary.

The earliest report of VSV as an inducer of cell death was the observation of toxic effects of double-stranded RNA derived from VSV infection in mice in 1973 [261]. However, the potential of VSV as an oncolytic virus (OV) was only demonstrated at the beginning of the 21st century. Stojdl *et al* (2000) observed that the VSV is readily grown to high titers and has a short replicative cycle, which are desirable properties of an OV [13]. It was further established that VSV rapidly replicates in and selectively kills a variety of human tumor cell lines, even in the presence of interferon doses that completely protect normal human primary cell cultures. VSV also has the ability to selectively induce cytolysis in transformed human cell lines *in vitro* [238]. Studies revealed the role of the PKR and p53 pathways in cells permissive to VSV [20, 238]. Later studies tested wild-type VSV and the attenuated AV1 and AV2 strains against a panel of tumor cell lines from the National Cancer Institute [11]: 80% of the tested lines were susceptible to VSV-induced oncolysis, particularly colon, ovarian, and renal carcinoma cells.

VSV oncolysis in leukemias has been well studied, and the results are controversial. Attenuated VSV strains AV1 and AV2, a heat-resistant (HR) strain, and a GFP-VSV were able to infect and kill leukemic cells in primary samples taken from patients with multiple myeloma (MM) [28]. Eleven of 12 leukemic cell lines tested were efficiently killed by all strains. It was further shown that VSV could replicate and cause extensive caspase-dependent oncolysis of primary ATL cells *ex vivo* [27]. However, naïve CD4+ lymphocytes were not killed by VSV. Chronic lymphocytic leukemia (CLL) cell lines permitted viral replication, but primary CLL cells were resistant to VSV replication and subsequent cell death. It was suggested that the inconsistency in VSV oncolysis could be due to the cell cycle arrest of CLL cells. More recently, it was confirmed that VSV oncolysis requires cell cycle entry and translation initiation [262]. These results support the hypothesis that VSV oncolysis in CLL cells is not effective because of the non-replicative nature of these cells. Nevertheless, VSV was effective against most leukemic cell lines tested.

Significant advances during the past decade have brought VSV-based therapeutics closer to reality. In 2006, a recombinant VSV ($VSV^{\Delta M51}$) was used successfully against multifocal and invasive glioma, prolonging survival of CD-1 nude mice, but did not affect normal cells *in vitro* [263]. A glioma-adapted vesicular stomatitis virus strain (VSVrp30a) had the capacity to kill glioblastoma cells *in vitro*, while sparing normal cells [18, 264-265]. Pre-clinical testing of VSV strains

should be completed during the next few years, and the use of VSV in clinical applications should soon become a reality.

APOPTOSIS

Upon viral infection and virus recognition by the host, several pathways are activated to stop viruses from spreading within the host. The existence of multiple viral mechanisms to block apoptosis supports the hypothesis that killing infected cells is the final pathway to eliminate infection [266]. Activation of the antiviral IFN pathway, and consequent production of type I IFN (α/β), are capable of inducing apoptosis in various systems. A variety of The IFN-regulated genes, such as tumor necrosis factor (TNF), tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), and CD95/FAS, that are actively involved in killing infected cells [267-268].

TNF is a family of soluble cytokines that mediate apoptosis and cell survival, among others functions [267-268]. TNF is primarily produced as a type II transmembrane protein, arranged in stable homotrimers upon activation by the immune system. TNF receptors (TNF-Rs) are membrane receptors activated by the immune system upon infection. They contain a homologous sequence, referred to as the death domain (DD), in their cytoplasmic tail. Two types of TNF-Rs have been described: TNF receptor type 1 (TNF-R1, CD120a, p55/60) and TNF receptor type 2 (TNF-R2, CD120b, p75/80). TNF-R1 is expressed in most

tissues, and can be activated by both membrane-bound and soluble trimeric forms of TNF. TNF-R2 is expressed only in cells of the immune system, and responds to the membrane-bound form of the TNF homotrimer. Activation of TNF-Rs leads to caspase activation via the adaptor proteins, TNF receptor-associated death domain (TRADD) protein and FAS-associated death domain protein (FADD). TNF activation can also activate cell survival and inflammatory pathways. Therefore, TNF activation has to be tightly controlled to avoid the development of diseases, especially autoimmune diseases [267-269].

TRAIL, also called Apo2 ligand, is a member of the TNF family of cytokines that promotes apoptosis via the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) in a caspase-8-dependent manner. TRAIL is expressed in a variety of cells, including spleen, liver, peripheral blood leukocytes, and activated T cells [270]. IFN-β stimulates expression of TRAIL in monocytes, dendritic cells, and macrophages; IFN-γ stimulates expression of TRAIL in monocytes, dendritic cells, and NK cells. Similar to TNF, TRAIL is a type II transmembrane protein, which can be bound to the cell surface or secreted as a signaling molecule [267, 270]. Engagement of DR4/DR5 by TRAIL results in recruitment of the death-inducing signaling complex (DISC), FADD, and caspase-8, which activates both the extrinsic and intrinsic apoptotic pathways. Activation of different apoptotic pathways is dependent on the cell type. In type I cells, the DISC-induced caspase

cascade is sufficient for the activation of effector caspases whereas type II cells require activation of the mitochondrial amplification loop.

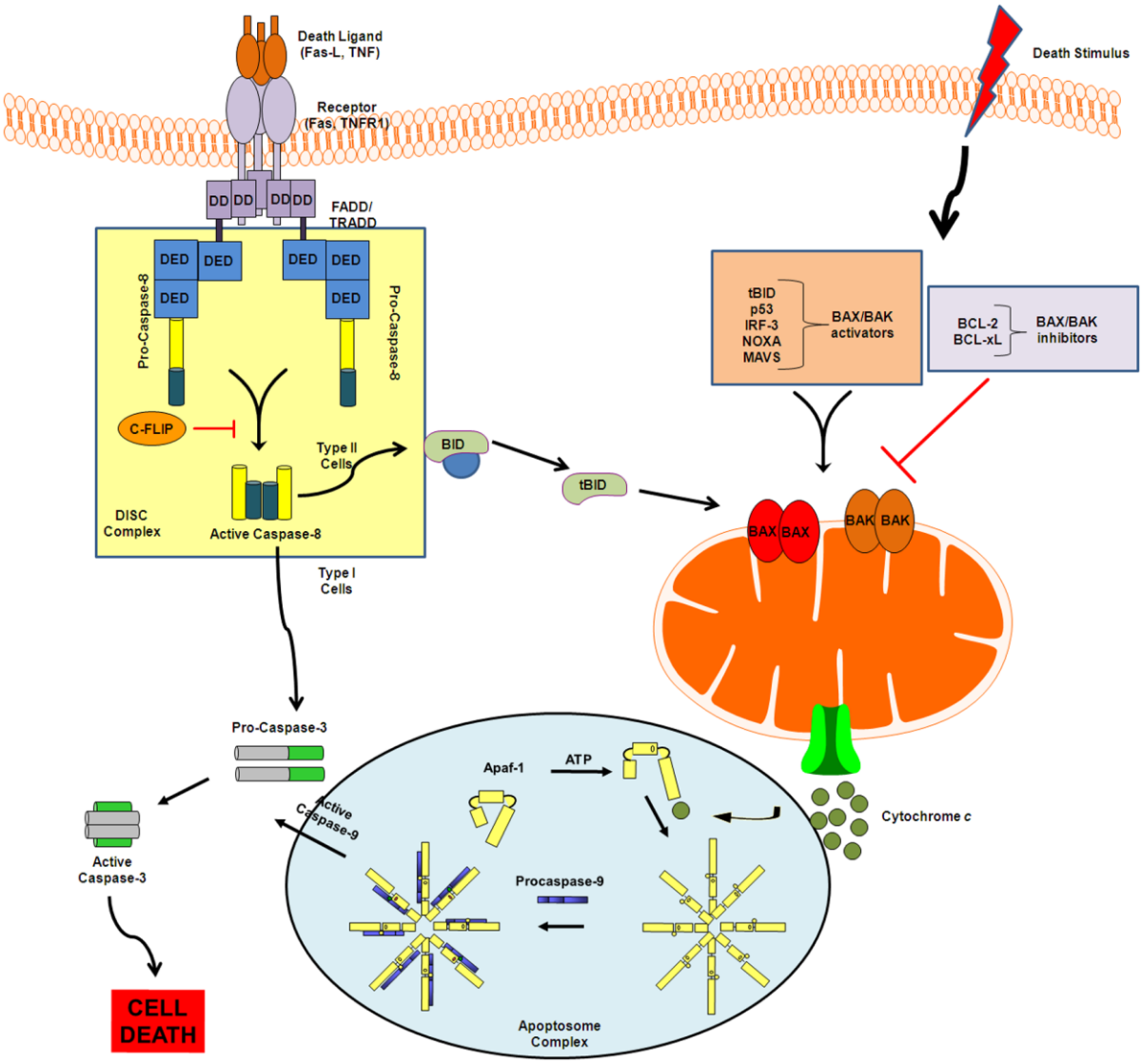
FAS ligand is another ubiquitously expressed protein of the TNF cytokine family [271]. Membrane-bound or soluble FAS ligand protein (FASL) binds to the FAS receptor which, similar to TNF and TRAIL, recruits the DISC, FADD, and caspase-8. As with TRAIL, activation of FAS signaling is sufficient for activation of the extrinsic pathway in type I cells, but amplification of signaling through the mitochondria is necessary in type II cells.

Extrinsic and intrinsic apoptosis

In response to danger signals, DNA damage, or senescence, cells undergo a process called apoptosis, or programmed cell death [272-273]. Apoptosis is a genetically programmed process of cell death, which maintains cellular homeostasis and tissue integrity in multicellular organisms. Apoptosis is initiated by external and internal stimuli, and involves specific morphological changes – cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation – eventually leading to break-up of the cell into "apoptotic bodies," and engulfment by macrophages or neighboring cells [272-273]. Although the morphological features of cells undergoing apoptosis are common to all cells, the molecular events that occur during apoptosis are specific to the apoptotic pathway triggered [272]. The molecular events fall in two groups: the death receptor-

mediated, extrinsic pathway and the mitochondrial-directed, intrinsic pathway. The biochemical changes associated with apoptosis include three main steps: activation of initiator caspases, mitochondrial protein release into the cytoplasm, and activation of effector caspases (Figure 2) [274-275].

Figure 2. Apoptosis pathway. Apoptotic cell death can be initiated through two main pathways: the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. The extrinsic pathway is mediated by caspase-8 whereas the intrinsic pathway is mediated by caspase-9. The death receptor pathway is activated by the adaptor protein FADD that couples with death receptors, such as FAS and TNFR1, to caspase-8. Cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP) prevents caspase-8 activation. Caspase-8 activation leads to caspase-3 cleavage, which initiates multiple proapoptotic processes. BCL-2 inhibits the loss of mitochondrial membrane potential, whereas BAX domains induce loss of mitochondrial membrane potential. Cytochrome c is released from the mitochondria and together with APAF1 and procaspase-9 form the apoptosome. In certain cell types, the two pathways are interconnected by truncated BID (tBID) that is formed when BID is cleaved by active caspase-8. tBID triggers BAX activation and translocation to the mitochondria.


Death Ligand
(Fas-L, TNF)
Receptor
(Fas, TNFR1)
Death Stimulus
DD DD DD DD
FADD/
TRADD
DED DED DED DED DED DED
Pro-Caspase-8
Pro-Caspase-8
C-FLIP
Type II
Cells
DISC
Complex
Active Caspase-8
BID
tBID
tBID
p53
IRF-3
NOXA
MAVS
BAX/BAK
activators
BCL-2
BCL-xL
BAX/BAK
inhibitors
BAX BAX
BAK BAK
Type I
Cells
Pro-Caspase-3
Active
Caspase-3
Active
Caspase-9
Apaf-1
ATP
Procaspase-9
Apoptosome
Complex
Cytochrome c
CELL
DEATH

Death receptor-mediated extrinsic pathway

In the death receptor-mediated, extrinsic pathway, ligands of death receptors, such as tumor necrosis factor (TNF) receptor superfamily, member 6 (FAS) and TNF ligand superfamily, member 10 (TRAIL), initiate a cascade of signals, which triggers the death-inducing signaling complex (DISC), resulting in activation of the 55 kDa precursor, procaspase-8. Activated caspase-8 triggers activation of caspase-3, which leads to cleavage of a number of substrates and induces the morphological changes characteristic of apoptosis and DNA fragmentation [273, 276].

Death receptors, which belong to the tumor necrosis factor receptor superfamily, include TNFR1, CD95/FAS DR3, DR6, TRAILR1, and R2, and have an extracellular domain and an intracellular death domain (DD) [277-279]. Upon engagement of the receptor, the intracellular DD interacts with adaptor proteins, such as FAS-associated death domain (FADD) and TNF receptor 1-associated protein (TRADD) (Figure 2) [275, 280]. Two signaling complexes are formed upon engagement of the death receptors: complex I and complex II [281-282]. Complex I is formed in the plasma membrane by TNFR1, the adaptor protein TRADD, RIP1 and TRAF-2. Activation of complex I leads to NF-κB activation. Once complex I leaves the death receptor, it forms the complex II which contains FADD, caspase-8, caspase-10, TRADD, RIP1 and TRAF2. The complex I

activation of NF-κB triggers prosurvival signals and when it's not activated, complex I activates complex II resulting in cell death [281].

The next step is DISC activation (Figure 2). Binding of TRADD adaptor protein is required for the recruitment of FADD and procaspase-8 for DISC formation.

DISC is formed by the oligomerized intracellular domain of the death receptor, the adaptor molecule, FADD or TRADD, procaspase-8, procaspase-10, and c-FLIP [280, 283]. C-Flip is an inhibitor of the DISC complex and caspase-8 via formation of catalytically inactive procaspase-8/c-FLIP heterodimers. Interactions between the molecules forming DISC are based on contact (Figure 2). The death domain of the death receptor interacts with the DD of the adaptor protein at the same time as the death effector domain (DED) of FADD or TRADD interacts with the N-terminal of the procaspases [283-284]. Procaspase-8 is activated at the death-inducing signaling complex (DISC) and both dimerization and proteolytic processing of procaspase-8 are required to form a caspase-8 dimer capable of initiating apoptosis [285-286]. Dimerization of procaspase-8 can produce enzymatically competent homodimeric precursors, which are more susceptible for processing than individual procaspase-8. c-FLIP prevents caspase activation by interacting with the DISC complex and blocking the activation and release of effector caspases. The main target of caspase-8 is the executioner, caspase-3, which upon activation, completes the execution of apoptosis (Figure 2) [287].

Caspases are a conserved family of proteases with vital roles in apoptosis [284, 288]. Caspase-3 is a key factor in the execution of apoptosis, and its activation is a hallmark of apoptosis [289-290]. Caspase-3 is present as a procaspase-3 within cells, and is responsible for the proteolytic cleavage of a number of key proteins involved in DNA degradation and cell death [290-291]. DNA fragmentation factor 45/inhibitor of caspase-activated DNAse (DFF45/ICAD) is a tight, non-covalent complex, endogenous chaperone/inhibitor [292]. Cleaved caspase-3 disrupts this complex and allows the free nuclease to dimerize into its catalytically competent form, 40-kDa caspase-3-activated nuclease/caspase-activated DNase (DFF40/CAD), which is responsible for cleavage of double-stranded DNA in apoptosis. Proteins, such as poly (ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), and U1-70kD, are involved in DNA repair [284, 290-291]. These proteins are targeted by activated caspase-3, resulting in their inactivation and ultimately leading to DNA degradation. Caspase-3 cleavage can also be involved in the amplification of caspase-8 activation during FAS signaling [293]. In some cell types, such as hepatocytes, activation of DISC and caspase-8 is generally not sufficient for full activation of downstream caspases; therefore, amplification of the death receptor pathway can be triggered by a mitochondrial step [294-295]. The BH3-only protein BID is the link between the extrinsic and the intrinsic mitochondrial pathways (Figure 2). Apical caspase-8 is able to cleave cytosolic BID, releasing its truncated form (tBID), which is translocated to the mitochondria. Once at the mitochondria, tBID induces

BAX/BAK-dependent permeabilization of the mitochondrial outer membrane, release of cytochrome *c*, formation of the apoptosome, and activation of the executioner caspase-3.

Intrinsic Pathway

In contrast to the extrinsic pathway, which mediates apoptosis through a subset of death signals at the plasma membrane, the intrinsic apoptotic pathway is initiated from within the cell by severe cell stresses, such as DNA damage, defects in the cell cycle, cell detachment from the extracellular matrix, hypoxia, and oxidative stress [272-273, 276]. The intrinsic pathway involves the mitochondria, and release of cytochrome *c* from the mitochondria into the cytoplasm is the major step implicated in full activation of this pathway. Tight control of the proteins involved in mitochondrial permeabilization is crucial to avoid impairment of the intrinsic apoptotic pathway and consequent disturbance of homeostasis and cell repair.

Mitochondrial outer membrane permeabilization (MOMP) is a pivotal step for initiation of the intrinsic pathway [296-298]. Pro- and antiapoptotic proteins from the B-cell lymphoma 2 (BCL-2) family strictly regulate the intrinsic apoptotic pathway by balancing each other upon upstream survival and distress signals (Figure 2) [299-303]. Proteins of the BCL-2 family have in common the presence of conserved BCL-2 homology (BH) domains, and are divided in two functional

groups: the survival, or antiapoptotic, group and the cell death inducer, or proapoptotic, group [55, 299, 304]. The antiapoptotic group includes BCL-2, BCL-xL, BCL-w, MCL-1, and A1 proteins. The proapoptotic group is subdivided into multi-domain proteins (BAX, BAK, and BOK), or BH3-only proteins (BIM, BAD, BID, BIK, BLK, HRK, NOXA, and PUMA). The first BH3-only protein discovered was BIK (BCL-2 interacting killer) [305]. Cell death activities of BAK, BIM, BID, BOD, and HRK were discovered around the same time through interaction experiments with the anti-apoptotic proteins, BCL-2, MCL-1, and BCL-xL [306-311].

Originally thought to be a growth-promoting oncogene, BCL-2 protein was instead found to enhance cell survival and interfere with apoptosis [312-314]. BCL-2 protein, the prototype member of the BCL-2 family, contains all the 4 BCL-2 homology domains (BH1–BH4) and mediates interactions with other family members [42, 44]. An important feature of BCL-2 is the presence of a hydrophobic groove, formed by the BH1–BH3 domains, which can bind the BH3 alpha-helix of proapoptotic proteins [315-316]. The ability to bind to the BH3 domain of other family members allows BCL-2 to sequester the pro-apoptotic proteins, BAX and BAK, obstructing their function and promoting cell survival [317-320].

Each BH3-only protein is triggered by specific apoptotic stimuli. For example, BAD responds to cytokine deprivation, while BIM is associated with growth factor withdrawal in B and T lymphocytes, and responds to ceramide and airborne particulate matter-induced apoptosis in alveolar epithelial cells [298, 321-323]. BID can be activated by a broad range of stimuli, including FAS-associated stimuli, lethal glutamate concentrations, or DNA damage-induced apoptosis (Figure 2) [271, 324-326]. BID is susceptible to proteolytic cleavage by caspases, calpains, granzyme B, and cathepsins [327-331].

BID is probably the most studied BH3-only protein, due its function as the connecting point between the extrinsic and intrinsic apoptotic pathways. In the late 1990s, several studies demonstrated that BID mediates a mitochondrial amplification loop of the death receptor signaling pathway, which results in the release of cytochrome *c* and the induction of cell death [332-337]. In 1996, Wang *et al.* demonstrated that BID could counteract protection by BCL-2, as well as induce apoptosis, by interacting with BAX [338]. BID can bind to BCL-2 or BAX monomers, but not to BCL-2/BAX heterodimers. NOXA is another BH3-only protein that is capable of combating the protection caused by antiapoptotic proteins. NOXA was first identified as a highly expressed gene in an ATL-derived tumor T-cell line (IKD cells) [339]. Ten years later, NOXA was suggested to be a mediator in p53-induced apoptosis [340]. More recently, it was discovered that NOXA is upregulated upon viral and dsRNA signals and

subsequently induces cell death [60-61, 341]. NOXA binds to MCL-1 [342-344] and, to a lesser extent, to BCL-2 and BCL-xL [340]. Binding with NOXA inhibits the antiapoptotic function of these proteins. Although NOXA has been demonstrated to be important in the induction of mitochondrial apoptosis [345-347], how NOXA stimulates cell death is still not well understood [348-349]. The most common role of NOXA is to bind antiapoptotic proteins, release sequestered proapoptotic proteins, and further induce mitochondrial permeabilization, cytochrome *c* release, and cell death (Figure 2) [340, 342-344, 350]. However, a few studies have suggested that NOXA can interact with BAX to induce pore formation in the mitochondrial membrane and cell death [351-352]. Schuler *et al* (2003) demonstrated that expression of NOXA protein occurs prior to BAX translocation to the mitochondria and cytochrome *c* release into the cytosol [352]. Moreover, NOXA association with BAX was demonstrated, in transfected cells, upon viral infection and dsRNA treatment [61].

The functional blockage of antiapoptotic proteins caused by BH3-only proteins allows proapoptotic proteins, such as BAX, to form oligomers at the mitochondrial membrane [55, 353-356]. This permeabilizes the membrane and triggers release of cytochrome *c* into the cytoplasm (Figure 2). Activation of BAX during apoptosis usually does not require an increase in transcription of its gene. BID and PUMA, but not BAD proteins, can bind to the first α helix (Hα1) of BAX and promote the conformational changes necessary for activation [357-

360]. BAX remains in the cytoplasm, with its putative tail-anchor sequence sequestered in a hydrophobic binding pocket on the surface of the protein, until induction of cell death [298, 361]. Once activated, conformational changes provoke BAX oligomerization: hidden domains become exposed, uncovering specific hydrophobic domains and increasing the affinity of BAX for the mitochondrial membrane (Figure 2) [298, 362]. Insertion of BAX oligomers into the mitochondrial membrane are believed to promote decrease of the mitochondrial membrane potential and swelling of the mitochondria. Ultimately, the outer mitochondrial membrane ruptures, causing a massive release of cytochrome *c*, which is normally located in the space between the outer and inner mitochondrial membranes [298, 361-365].

Permeabilization of the mitochondria induces formation of the apoptosome, a multiprotein complex containing cytochrome *c*, apoptotic protease-activating factor 1 (APAF-1) and procaspase-9 (Figure 2) [272, 366-367]. Once released, cytochrome *c* binds to APAF-1, which undergoes conformational changes to form the apoptosome and activate pro-caspase-9. Subsequently, activated caspase-9 cleaves effector caspases-3 and -7, which are responsible for proteolytic cleavage of many downstream death proteins, such as PARP and DFF45/ICAD [291-292]. Activation of caspase-3 can also activate the mitochondrial loop, in which caspase-3 amplifies BAX translocation to the mitochondria [294-296].

VSV-induced apoptosis

Induction of apoptosis by viruses is a common approach to overcoming the host, and VSV infection causes cytopathic effects (CPE) consistent with the induction of apoptotic pathways [368]. VSV induces apoptosis via distinct mechanisms in a cell-dependent manner by activating multiple apoptosis-inducing pathways. VSV induces the death-receptor apoptotic pathway or the mitochondrial extrinsic pathway. VSV-induced apoptosis via the extrinsic pathway is mediated by FAS, PKR and the FAS adaptor protein DAXX rather than FADD [234, 369-370]. Levels of FAS protein were demonstrated to be elevated in VSV-infected L929 cells [234]. Activation of FAS signaling, probably through PKR, triggers translocation of FAS to the cell surface where it forms the DISC complex. Moreover, PKR activation induces translational inhibition and NF-kB activation through the IkB kinase complex [370-371]. Caspase-8 is then recruited by the death-induced signaling complexes (DISC) dependent on DAXX where it is activated [234, 369]. Finally, activated caspase-8 induces activation of caspase-3, which promotes activation of several death signals, including DNA fragmentation and poly (ADP ribose) polymerase (PARP) cleavage [31, 176, 372]. Cross-talk between the extrinsic and intrinsic apoptotic pathway has been shown to occur in VSV-infected cells [29]. In this case, sensing of virus by the death receptors activates pro-caspase-8 that in turn activates the intrinsic pathway via Bid cleavage which activates BAX/BAK promoting their oligomerization at the mitochondria membrane. VSV induction of intrinsic apoptosis can also starts at

virus recognition by the cytosolic sensor RIG-I which recognizes VSV's 5′-triphosphates RNA [373]. Upon virus recognition, RIG-I recruits the adaptor protein MAVS. Although actual demonstration of RIG-I/MAVS-induced apoptosis in VSV infected cells, this pathway is known to induce the transcription of the pro-apoptotic BH3-only protein NOXA in dsRNA virus infected cells, which suggests that this mechanism might possibly happen upon VSV infection [374]. Induction of NOXA in VSV-infected cells was reported in different studies which showed that NOXA is induced independently of p53 but dependent of IRF-3 [60-61]. In 2000, Heylbroeck *et al* demonstrated that active IRF-3 can induce cell death independent of type I IFN production. The authors showed that IRF-3 mediates binding to cytosolic BAX to induce BAX activation; however, it remained unclear if IRF-3 would displace the pro-survival BCL-2 protein from BAX or whether IRF-3 would directly activate BAX [375]. Years later, activation of the RIG-I/MAVS pathway was demonstrated to lead to activation of IRF-3 which binds to the IRFE elements within the NOXA promoter [60-61]. In these studies, it was suggested that IRF-3-induced NOXA could bind to BAX leading to BAX activation and oligomerization at the mitochondrial membrane. Oligomerization of BAX results in its activation on the mitochondrial membrane, triggering pore formation and subsequent release of cytochrome *c* into the cytosol [299, 355]. In the cytosol, cytochrome *c* forms the apoptosome with APAF-1 and caspase-9, which is responsible for cleavage of caspase- 3 and consequent activation of death signals [30-31, 376]. Interactions among proteins of the BCL-2

family are important in maintaining the balance between cell life and death [56, 299, 377]. In the same way that proapoptotic proteins are important in VSV-induced cell death [29, 378-379], imbalance of anti-apoptotic proteins, such as BCL-2, MCL-1 and BCL-xL, can impair apoptosis in cells infected with VSV [39, 379-380]. Virus infection can also lead to intrinsic apoptotic pathway mediated by the p53 transcription family (p53, p63 and p73 proteins). p53 up-regulates pro-apoptotic genes such as BAX and suppress anti-apoptotic genes such as BCL-2, by transcriptional activation or repression, thus altering the relative quantities of BAX to BCL-2 and shifting the balance towards apoptosis [381]. However, evidence of p53-induced apoptosis in VSV-infected cells has yet to be clarified as conflicting studies have shown VSV-induced p53-dependent apoptosis [382] and that VSV-induced down-regulation of p53, rather than its activation, seams to sensitize cells to apoptosis [379, 383]. What becomes clear from the available literature on VSV-induced apoptosis is that despite the VSV strain and how apoptosis signal is started the intrinsic mitochondrial apoptotic pathway is required for full activation of apoptosis by VSV with BAX and BAK as the central proteins involved in permeabilization of the mitochondria and release of cytochrome *c*.

BCL-2 OVEREXPRESSION AND CANCER

The cell death pathway responds to both normal and pathologic stimuli, and aberrancies in apoptosis have been associated with human diseases, including

autoimmunity, cancer, immune deficiency, and neurodegenerative disorders [40-44, 295, 303, 384-386]. Elevated expression of prosurvival proteins, such as BCL-2, is a common deregulator of programmed cell death. BCL-2 overexpression inhibits the mitochondrial apoptotic pathway, which can account for drug resistance and poor clinical prognosis in several types of cancer, especially B-cell malignancies [40, 43-44, 317, 387]. In fetal tissues, BCL-2 is widely expressed in the thymus, lymph nodes, and spleen, but decreases in adulthood and becomes confined to cells that are rapidly dividing and differentiating [44, 388].

The B-cell lymphoma 2 (BCL-2) protein was discovered during cloning of the t(14;18) translocation breakpoint, which generated unusual high levels of BCL-2 protein in follicular lymphoma [386, 389]. The first characterization of BCL-2 overexpression was associated with chromosomal rearrangement t(14:18) in human follicular non-Hodgkins lymphoma (NHL) [40, 390]. Overexpression of the BCL-2 protein was also associated with exacerbated amplification of the BCL-2 gene in small cell lung carcinoma [391] and diffuse large B-cell lymphoma [392]. The importance of the relationship between t(14;18) translocation and BCL-2 overexpression declined when patients were found who presented the translocation, but did not have elevated levels of BCL-2, or who presented high levels of BCL-2, but did not have the chromosomal rearrangement. High BCL-2 levels has also been attributed to inactivation of the microRNAs (miRs), miR15 and miR16, in chronic lymphocytic leukemia (CLL) [40, 393-

395]. Moreover, the epigenetic modification hypomethylation, in neuroblastoma cells, has been reported to cause excessive expression of BCL-2 protein [390, 396].

The realization that BCL-2 overexpression could both act as an oncogene and was associated with impairment of apoptosis led to the hypothesis that defects in apoptosis are critical in tumor development and maintenance. Indeed, the role of BCL-2 in malignancies has been the focus of many studies. Ikegaki *et al* demonstrated that 80% of small cell lung carcinoma (SCLC) cell lines expressed high levels of BCL-2 [391]. High levels of BCL-2 were later associated with invasive and metastatic potentials in non-small cell lung carcinoma (NSCLC) [397] and melanoma cells [398]. Antiapoptotic BCL-2 was overexpressed in 95% of CLL cases [399]. Furthermore, BCL-2 overexpression was related to tumor aggressiveness, rate of cell proliferation, and primary resistance to chemotherapy [400-402]. The significant relation of BCL-2 and resistance chemotherapy supports the postulate that alterations in the BCL-2 pathway may influence clinical outcome by deregulation of apoptosis. Therefore, antiapoptotic proteins, such as BCL-2, offer an attractive, but challenging, target for the development of anticancer agents, which could improve tumor sensitivity to new and current treatments.

CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in adults over 50 years of age in the U.S. and Europe, and is twice as common in men as in women [33-34, 403]. Familial occurrence of CLL exists; cases have been described in first and second degree relatives of CLL patients [404]. The course of this disease differs among individuals: some live for decades after the diagnosis and die from non-cancer-related causes; others die rapidly, regardless of treatment [403, 405-407]. Median patient survival is 10 to 25 years, but aggressive cases have a prognosis of less than 8 years of survival, and only 1-2 years in some cases [407-408].

CLL is characterized by extensive accumulation of small, mature looking, malignant lymphocytes in peripheral blood and bone marrow [33, 38, 405, 409]. Immune deficiency and thrombocytopenia commonly develop during the course of CLL. Frequent symptoms are erythroderma, adenopathy, peripheral cytopenias, fever, weight loss, and night sweats. Later in disease development, neoplastic cells infiltrate the bone marrow, and secondary infections develop, such as bacteria, herpes zoster, and herpes simplex.

Diagnostic criteria for CLL were formulated independently by the National Cancer Institute Working Group (NCI-WG) [410] and by the International Workshop on CLL (IWCLL) [411]. The criteria were recently reformulated,

reflecting considerable progress in defining new prognostic markers and diagnostic parameters [412-413]. Diagnosis of CLL is now based on blood counts and immunophenotyping: absolute lymphocytosis in peripheral blood (NCI: >5 x 10^9 L^{-1}; IWCLL: 10 x10^9 L^{-1}) and characteristic immunophenotype ($CD5^+$, $CD19^+$, $CD20^+$, $CD23^+$). The majority of lymphocytes usually have a small and mature appearance. Bone marrow infiltration is observed, with at least 30% of lymphocytes in bone marrow aspirate. CLL does not usually form tumor masses. Therefore, diagnosis and prognosis must depend on other information, such as laboratory results and imaging tests. Microscopic examination confirms lymphocytosis and flow cytometry analyzes clonality. Lymph node biopsy differentiates CLL from small lymphocytic leukemia (SLL) [403-404, 412]. Although not necessary for the diagnosis or staging of CLL, molecular testing, such as chromosomal evaluation, IgVH status, and ZAP-70 and CD38 expression, may help predict prognosis or clinical course [408, 414-416].

Classification of CLL follows one of two cytological staging systems, Rai Classification [417] and Binet Staging [413, 418]. According to these systems, CLL patients can be divided in three main groups. The first group is Binet stage A or Rai stage 0, in which patients are asymptomatic, and progress of the illness varies among individuals. Patients in this stage are followed by their physicians; treatment is not necessary unless progression is noted. The second group is Binet stage B or Rai stage I/II, in which patients present an intermediate risk, and

treatment is necessary if progression is noted. The third group, Binet stage C or Rai stage III/IV, has higher risk. Treatment is immediate, with aggressive combination regimens. Allogeneic transplant can be considered as the only potentially curative therapy.

MOLECULAR BIOLOGY OF CLL

CLL is an incurable disease, and the available treatments are mildly effective to ineffective [407-408, 419]. The causes are unknown, but the molecular environment of CLL cells plays a crucial role in the development of pathogenesis [405, 407]. Typically, CLL cells express the antigens CD19, CD5, and CD23, on their surface [33, 403-404]. Low expression or absence of CD22, FMC7, and CD79b are also common molecular markers of CLL [33-34, 404, 413, 420-421]. Although certain parameters, such as immunoglobulin mutational status, are not used for diagnosis they are useful for predicting the clinical course in individual cases

A few prognostic markers have been identified in CLL cells: CD38, ZAP-70, and mutational status of the immunoglobulin heavy-chain variable region (IgVH) have been added to the Rai and Binet staging systems. CD38 is a transmembrane glycoprotein with enzymatic activity, which catalyzes the synthesis of cyclic ADP-ribose (cADPR) [422-423]. CD38 is engaged by its ligand, CD31, which induces a signaling cascade that leads to cell proliferation. CD38/CD31 cross-talk

controls a critical activation pathway in CLL proliferation and survival. $CD38^+$ patients have a minimum of 30% of CD38 expression, which is detected by flow cytometry [403, 424-425], and progress more rapidly to advanced Rai stages than $CD38^-$ patients [426]. Positivity for CD38 is associated with a short time from diagnosis to treatment and a low survival rate.

ZAP-70 (zeta-associated protein with a molecular weight of 70 kD) is a molecule that transmits a signal from a T-cell receptor to downstream pathways. Most B cells lack ZAP-70 and use the related spleen tyrosine kinase (Syk) for signal transduction [427]. The ZAP-70 gene is the most relevant gene distinguishing the mutated and unmutated IgVH subgroups of CLL [428-429]. Ninety-three percent of patients could be correctly classified into the subtypes, based on ZAP70 expression [430]. Expression of ZAP-70 partially determines the capability of CLL cells to respond to antigenic stimulation [431-432]. In particular, ZAP-70 expression is associated with activation of serine/threonine kinase-protein kinase B (AKT) and elk-related tyrosine kinase (ERK), and subsequent induction of several survival proteins, including BCL-2 and MCL-1 [414, 427]. ZAP70 is a strong prognostic indicator in CLL: high presence of ZAP-70 positive cells is predictive of more aggressive disease, with low survival compared to patients with low ZAP-70 expression [403-404, 408, 414-415, 431, 433].

Immunoglobulins (Ig) are B-cell receptors (BCR) present on the surface of B-cells. BCRs mediate B-cell signaling and engage any possible antigen. The diversity of immunoglobulins enables recognition of the many antigens that a host must fight in its lifetime. The immunoglobulin heavy chain (IgV) is the region responsible for recognition and binding of antibodies to their target markers. The hypervariable (HV) region within the heavy chain accounts for specificity and affinity [434]. IgVH genes determine the quality of binding between the antigen and the membrane-bound Ig receptor, which is crucial for clonal survival during B cell development, and for antigenic stimulation of mature B cells [435]. Signaling by the Ig receptor rescues pre-B cells and proliferating mature B cells from apoptotic cell death [414, 435]. Somatic mutations in the IgVH gene have prognostic significance in CLL: patients with unmutated IgVH genes have higher likelihood of early requirement for treatment, greater possibility of resistance to therapy, and inferior survival compared to patients with mutated IgVH genes [408, 415, 424, 433, 436-437].

TREATMENT OF CLL

Physicians first assess a patient's risk factors to evaluate their need of treatment. Although the Rai and Binet stages allow physicians to design therapies based on the risk of disease progression, they do not predict treatment outcome [403, 438]. Relapse is a common aspect of CLL treatment, and patients with long remissions require different approaches [38, 439]. To date, five standard treatments are

available to CLL patients: 1) watchful waiting, where patients are closely monitored without any treatment until symptoms appear; 2) radiation therapy; 3) surgical removal of the spleen; 4) chemotherapy to kill cancer cells or to stop cell division; and 5) targeted therapies that identify and attack cancer cells without harming normal cells.

Alkylating agents and purine analogues used in chemotherapy regimens are among the first choices in treatment of CLL. Properties of sulphur mustards observed during the World War II identified alkylating agents as possible therapy against cancer [440-442]. Mustard agents caused nausea, vomiting, and myelosuppression, associated with high proliferative rates of some organ tissues. Malignancies that are highly proliferative, like leukemia and lymphomas, could be susceptible to these agents. Alkylating agents add alkyl groups to many electronegative groups via three main mechanisms [440, 443]. First, alkylating agents can attach alkyl groups to DNA bases, resulting in DNA fragmentation by repair enzymes attempting to replace the alkylated bases. Fragmentation prevents DNA synthesis and RNA transcription from the affected DNA. Second, alkylating agents can cause formation of cross-links between the atoms in DNA, preventing separation of the DNA strands during synthesis or transcription. Third, alkylating agents can induce nucleotide mispairing and subsequent mutations.

Cyclophosphamide (CTX) and chlorambucil (CLB) are two frequently used alkylating agents. Both are cytotoxic, forming intra- and inter-strand DNA and DNA-protein cross-links, inhibiting DNA replication, and inducing cell death by apoptosis. CTX and CLB were the first choice of treatment for CLL starting in the early 1950s [403, 439], but the risk of developing secondary acute myeloid leukemia, which increases toxicity and reduces effectiveness, showed the need for more effective alternatives.

Purine analogues provide significant overall improvement in CLL and are currently the first option for treatment [438, 444]. Purine analogues are cytotoxic agents with chemical structures similar to adenosine and deoxyadenosine [445-446]. Uptake by cells is dependent on nucleoside transporters. Purine analogues require biological activation by phosphorylation, and their cytotoxicity depends on accumulation of the derivative triphosphate forms in the cell. For example, fludarabine, the leading drug in this category, is converted to the triphosphate form, F-ara-A, which enters proliferating cells and is incorporated into DNA by DNA polymerases. Once incorporated, F-ara-A causes inhibition of ribonucleotide reductase and DNA polymerase, which decreases DNA synthesis [405, 447-450]. Although patient responses to purine analogues as single agents can reach 50-60% [403, 405, 444, 447, 451-452], combination regimens, such as fludarabine and cyclophosphamide (FC) or fludarabine, rituximab, and cyclophosphamide (FCR) provide even better results [438, 447, 451, 453-454].

Immunotherapy has recently demonstrated significant potential in CLL treatment [419]. Rituximab, a monoclonal antibody, targets the transmembrane protein, CD20, of B cells [455]. CD20 is expressed in 90% of mature B-cell leukemias [405, 438]. Binding of Rituximab to CD20 leads to significant depletion of peripheral B cells [456-459]. CLL patients, Rituximab is associated with TNF-α release, which causes CLL proliferation and inhibits apoptosis and therefore, has poor efficacy against CLL as a single agent [403], but combinations with fludarabine or etanercept showed efficient results and favorable safety profiles. The F-ara-A form of fludarabine enters proliferating cells and is incorporated into DNA as a false nucleotide which inhibits DNA synthesis causing cytotoxicity and apoptosis. Incorporation of F-Ara-A into replicating DNA of dividing cells may trigger pro-apoptotic signaling mechanisms thus increasing the sensitivity of B-CLL cells to rituximab-mediated caspase-3 activation (A. Furlan et al 2010). Etanercept is a TNF-α antagonist that diminishes Rituximab toxicity by inhibiting the effects of Rituximab-induced TNF-induced proliferation of CLL cells [460].

Alemtuzumab is an anti-CD52 monoclonal antibody approved for the treatment of patients with relapsed CLL [405, 461]. Results of clinical trials suggest that alemtuzumab therapy results in high response rates and durable remissions [462]. A regimen using alemtuzumab and rituximab showed effectiveness for early treatment of patients with high risk CLL [463].

Although combination of chemotherapy and immunotherapy regimens improves the prognosis of CLL, there is a continued need for new, more effective, therapies. CLL is characterized by B lymphocytes that accumulate by escaping apoptosis, rather than by increased proliferation [35, 438]. Deregulated expression of proteins involved in apoptosis, such as BCL-2, leading to increased cell survival, is frequently diagnosed in CLL cells. High levels of antiapoptotic proteins are associated with poor response to chemotherapy and advanced stages of CLL [35, 464-467]. Blockage of BCL-2 activity by molecular-targeted therapy may abrogate the survival effect that is conferred by CLL. Thus, small molecule BCL-2 inhibitors may provide new and more effective treatment options for CLL, with minimal toxicity.

To date, three types of small molecules that might be used to fight overexpression of pro-survival proteins have been examined: natural compounds, antisense oligonucleotides, and BH3 mimetic agents. Natural compounds, including antimycin A which binds to BCL-2 [468-471] and chelerythrine an alkaloid that disrupts BCL-xL/BAX interaction [52, 472-475], were reported to induce cell death in malignancies, such as mesothelioma and CLL. However, other studies showed that cell-death activity of these compounds was independent of BAX and BAK [476]. Moreover, weak binding affinity of these compounds to the BCL-2

family of proteins suggested that binding to BCL-2 proteins is not related to their cytotoxicity.

Antisense oligonucleotides (ASOs) are small-interfering RNA (siRNA) sequences that target the open reading frame of BCL-2 and MCL-1 mRNA sequences [477-480]. Binding of (ASOs) blocks mRNA translation, which inhibits the expression of its gene product [481]. Specific down-regulation of expression of antiapoptotic proteins leads to increased apoptosis [480, 482-483]. Phase I/II and III clinical trials confirmed the effectiveness of this treatment [481, 484-486]. Unfortunaly, delivery of ASOs is a major obstacle that must be overcome for successful implementation of antisense technology. To date, liposome-based delivery, electroporation, cationic liposomes, and receptor-mediated endocytosis have been explored, but the efficient delivery of the ASO molecules into target cells remains challenging [487-489].

BH3 mimetic drugs, a relatively new category of small molecule inhibitors that act as anti-cancer agents, are gaining recognition due to success in xenograft models and clinical trials [476, 490-492]. BH3-only mimetics bind to the surface pocket of proapoptotic proteins, such as BCL-2, BCl-xL, and MCL-1, disrupting the association between pro- and antiapoptotic proteins [493-495]. HA14-1, the first BCL-2 inhibitor described, was identified by computer screening, of a chemical library, combined with cell-based assays [496]. HA14-1 binds to the -2

surface pocket of BCL-2 [496-497] and MCL-1 [498], disrupting their interaction with BAK or BAX. HA14-1 enhances the cytotoxic effects of a number of anti-cancer agents [49, 499-503]. However, HA14-1 induces apoptosis at relatively high concentrations (5-50 μM) compared to recently developed inhibitors, which are effective in the nanomolecular range. Furthermore, overexpression of BCL-2 confers modest resistance to HA14-1 [504]. Therefore, this agent is not a therapeutic candidate for malignancies, such as CLL, that overexpress BCL-2.

A small molecule inhibitor, ABT-737, was discovered by Abbott Laboratories through screening strategies that used nuclear magnetic resonance, structure-based design, and combinatorial chemical synthesis [505]. ABT-737 has a high affinity for BCL-2 and BCL-xL, but does not bind MCL-1, so that many cell types are refractory to its effects [505-506]. ABT-737 disrupts the BCL-2/BAX complex and BAK-dependent activation of the intrinsic apoptotic pathway [48], and elevated NOXA levels are directly correlated with sensitivity to ABT-737 [507-508]. Thus, ABT-737 and its orally active analog, ABT-263 [509-510], are potent inducers of cell death. *In vitro* and *in vivo* experiments demonstrated toxicity of ABT-737 as a single agent to leukemias and lymphomas [46, 50, 511-512]. Preclinical studies demonstrated activity of ABT-263 against acute lymphoblastic leukemia (ALL) cell lines, but only limited single agent activity against solid tumor cell lines. Combination therapies using ABT-737 demonstrated high effectiveness against leukemias and non–small cell lung cancer

[512-514]. ABT-737 was only partially effective against CLL cells *ex vivo*: upregulation of BCL-xL and BCL-2 induced 1000-fold resistance to chemotherapy in CLL cells [515]. However, the use of ABT-737 in combination with chemotherapic agents improved response rates in CLL [516]. ABT-737 and ABT-263 are currently undergoing Phase I/II clinical trials [495, 517].

Another third molecule inhibitor, obatoclax mesylate (GX15-070), is an indole bipyrrole compound and a pan-inhibitor of the prosurvival proteins in the BCL-2 family. Obatoclax engages at the hydrophobic pocket within the BH3 binding groove of BCL-2, facilitating BAX/BAK-dependent apoptosis [53, 517-518]. Obatoclax was discovered through a high-throughput screen of natural compound libraries. Studies involving solid tumors [519-522] and hematological malignancies [45, 58-59, 523] demonstrated that obatoclax is effective as a single agent. Obatoclax also proved to exert clinical activity as single-agent in CLL patients [59], and was well tolerated by patients with myelodysplasia [58]. Obatoclax has an even more promising ability to augment the effectiveness of standard therapies [45, 519-521, 523]. Obatoclax is currently being tested as a single agent and in combination with bortezomib and docetaxel in over sixteen Phase I and I/II clinical trials for acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), CLL, and Hodgkin's lymphoma. (www.geminx.com/en/clinical/active.php). Bortezomib induces accumulation of the antiapoptotic protein MCL-1 and combination with

Obatoclax overcomes MCL-1-mediated resistance to Bortezomib [45, 522]. Docetaxel is a well established anti-mitotic chemotherapeutic drug. Docetaxel promoting and stabilize microtubule assembly, while preventing physiological microtubule depolymerisation and disassembly which leads to a significant decrease in free tubulin, needed for microtubule formation and results in inhibition of mitotic cell division. Also, docetaxel leads to BCL-2 phosphorylation which inhibits apoptosis. Therefore, inhibition of BCL-2 function by Obatoclax reverses docetaxel-mediated inhibition of apoptosis [524].

Since overexpression of antiapoptotic proteins is a challenge to therapeutic agents, the ability of obatoclax to overcome resistance in malignancies like CLL [39, 522] is highly desirable. BCL-2 overexpression in CLL provided an excellent model to test the impact of BCL-2 antagonism. Obatoclax was specifically designed to inhibit all of the anti-apoptotic members of the BCL-2 protein family, and was the first small molecule, pan-inhibitor of BCL-2 proteins tested in clinical trials. Therefore, obatoclax might be the compound of choice in challenging diseases such as CLL.

CHAPTER 2

Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary Chronic Lymphocytic Leukemia to VSV-induced oncolysis

Preface to Chapter 2

Previously, study from our group demonstrated that Vesicular Stomatitis Virus is not able to induce apoptosis in CLL cells. Defects in the apoptotic pathway, specifically, overexpression of the BCL-2 protein has been closely associated with resistance to anticancer therapy. As VSV induces cell death through the mitochondrial apoptotic pathway, we postulated that the same defects in the apoptotic pathway responsible for treatment resistance in CLL could also be accountable for the VSV failure in inducing cell death in these cells. Therefore, we sought to investigate if a BCL-2 inhibitor would be able to restore the apoptotic signal in CLL cells and enable VSV oncolysis.

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by clonal accumulation of $CD5^+/CD19^+$ B-lymphocytes that are arrested in the G0/G1 phase of the cell cycle and fail to undergo apoptosis because of overexpression of the anti-apoptotic B-cell CLL/lymphoma 2 (BCL-2) protein. Oncolytic viruses such as Vesicular stomatitis virus (VSV) have emerged as potential anticancer agents that selectively target and kill malignant cells via the intrinsic mitochondrial pathway. Although primary CLL cells are largely resistant to VSV oncolysis, we postulated that targeting the apoptotic pathway via inhibition of BCL-2 may sensitize CLL cells to VSV oncolysis. In the present study, we examined the capacity of EM20-25 - a small-molecule antagonist of the BCL-2 protein - to overcome CLL resistance to VSV oncolysis. We demonstrate a synergistic effect of the two treatments in primary *ex vivo* CLL cells (Combination index: CI=0.5; $p<0.0001$). In a direct comparison of peripheral blood mononuclear cells from healthy volunteers with primary CLL, the two agents combined showed a therapeutic index of 19-fold; furthermore, the combination of VSV and EM20-25 increased apoptotic cell death in Karpas-422 and Granta-519 B-lymphoma cell lines ($p<0.005$) via the intrinsic mitochondrial pathway. Mechanistically, EM20-25 blocked the ability of the BCL-2 to dimerize with pro-apoptotic BAX protein, thus sensitizing CLL to VSV oncolytic stress. Together, these data indicate that the use of BCL-2 inhibitors may improve VSV oncolysis in treatment-resistant

hematological malignancies such as CLL, with characterized defects in the apoptotic response.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in the Western hemisphere, accounting for up to 30% of all diagnosed leukemia. Characterized by a progressive accumulation of a monoclonal $CD5^+/CD19^+$ B-lymphocyte population in the peripheral blood, bone marrow, and lymphoid organs, as well as low levels of cell surface immunoglobulin, these cells ultimately acquire an aggressive and lethal phenotype [420]. Malignant B cells are arrested in G0/G1 phase of the cell cycle and fail to undergo apoptosis due to overexpression of B-cell CLL/lymphoma 2 (BCL-2) protein in malignant CLL cells [43, 525]. The antiapoptotic BCL-2 protein plays a key role in the control of the intrinsic mitochondrial pathway and promotes cell survival by inhibiting the function of proapoptotic proteins such as BAX and BAK [55, 300, 526]. Although chromosomal translocation events such as t(14:18) have been associated with BCL-2 overexpression in several types of follicular B-cell lymphomas, the mechanisms that mediate BCL-2 expression in CLL cells remain unclear [34, 55, 464]. Despite advances in cancer therapeutics, CLL disease remains resistant to existing treatments; the majority of therapies are palliative, with only a small percentage of patients achieving a complete response [403, 527].

Viral oncolytic therapy is a promising new strategy for cancer treatment [14, 528], involving the use of replication-competent viruses that specifically target and kill cancer cells, while sparing normal tissues. This selectivity is achieved by exploiting cell surface or intracellular aberrations in gene expression that arise

during the development of malignancies, and appear to favor cancer cell proliferation at the expense of the host antiviral program [528-530]. Vesicular stomatitis virus (VSV) is an enveloped, single stranded RNA virus member of the *Rhabdoviridae* family possessing intrinsic oncolytic properties [11, 13, 528]. Aspects of interferon (IFN) signaling and the action of downstream effectors including translational control are compromised in malignant cells, thus affording a cellular environment that facilitates viral replication and cell killing - uninterrupted by the host antiviral response [18]. Naturally attenuated VSV strains (termed AV1 and AV2) harboring mutations in the Matrix protein have a potentially greater therapeutic margin when compared to wild type VSV (49), because these attenuated strains fail to block nuclear to cytoplasmic transport of host mRNA – including IFN and cytokine mRNA – and therefore generate an antiviral response that contributes to a strong protective effect in normal tissue [17].

It has been generally accepted that VSV induces apoptosis in a caspase-3 and -9-dependent manner [11, 30]. Despite discrepancies about the particular involvement of either the intrinsic or extrinsic pathway in VSV-induced apoptosis [31, 234], the proapoptotic protein BAX represents the convergence point of VSV-mediated cell death, triggering mitochondrial membrane potential depolarization [29]. We previously reported [27] that primary *ex vivo* CLL cells are resistant to VSV-induced apoptosis; given the importance of mitochondrial

pathway in VSV oncolysis, we hypothesized that inhibition of BCL-2 function may restore activation of the intrinsic apoptotic pathway in VSV infected malignant CLL cells. Indeed, we demonstrate that primary CLL cells that are refractory to VSV-induced apoptosis can be rendered sensitive to VSV oncolysis by combination treatment with VSV-AV1 and a BCL-2 inhibitor. Impressively, our data also demonstrates that induction of apoptosis by combination treatment is not toxic for normal PBMCs, suggesting that the use of VSV and a BCL-2 inhibitor constitutes a promising, therapeutic approach for the treatment of chronic lymphocytic leukemia.

RESULTS

Primary CLL cells are resistant to VSV-AV1-induced apoptosis

Overexpression of the antiapoptotic BCL-2 protein has been associated with an apoptosis-resistant phenotype in CLL and other malignances [317, 464-465]. To analyze VSV-induced oncolysis in BCL-2 overexpressing malignant cells, primary CLL cells from seven patients were infected with a naturally attenuated variant of VSV (AV1) at 10 MOI, and by 96 h cells were analyzed for virus replication and apoptosis. All samples showed high levels of BCL-2 protein compared to samples from healthy volunteers (Figure 1a). Despite detectable viral replication in all samples (Figure 1c), VSV-AV1 did not induce significant apoptosis in the CLL samples assayed by Annexin-V staining (Figure 1b). Indeed, infection at higher MOIs of VSV-AV1 (10, 30 and 60 MOI) did not further

increase apoptosis (Figures 1d,e) suggesting that BCL-2 overexpression may play a role in resistance to VSV-induced apoptosis in CLL cells.

BCL-2 inhibitor EM20-25 sensitizes *ex vivo* CLL cells to VSV-AV1 oncolysis

Small organic molecules such as EM20-25 [52, 531] (Figure 2a), have been shown to sensitize apoptosis-resistant cells to cytotoxic drugs by binding to BCL-2 and disrupting interactions with proapoptotic proteins such as BAX and BAK. As such, investigations are underway to explore the use of small molecule inhibitors as therapeutic agents for the treatment of malignances that overexpress BCL-2 [49, 51, 532]. To examine the effect of EM20-25 on VSV oncolysis in CLL, primary human CLL cells from 7 different patients (CLL8-11, 13-15) were pre-treated with 10 μM of EM20-25 (IC_{50} approximately 20 μM – Figure 2b) and infected with VSV-AV1 (10 MOI). Pre-treatment with EM20-25 for 30 min dramatically diminished the resistance of CLL to VSV-induced apoptosis (Figure 3a). By 96 h post-infection the combination increased cell death more than 5-fold, compared to either VSV-AV1 or EM20-25 alone (1.1- and 1.4-fold respectively) (Figure 3b). Importantly, synergism - measured using Combination index: CI=0.5 - (see Methods) was achieved in all seven samples tested - ($p<0.0001$, compared with VSV-AV1 alone or $p<0.001$, EM20-25 alone) (Figure 3c). Induction of apoptosis by VSV-AV1/EM20-25 in malignant CLL was further confirmed by detection of cleaved caspase-3; the level of cleaved caspase-3 was 7 to 10-fold higher in cells treated with both VSV-AV1 and EM20-25 than in cells treated

with either agent alone (Figure 3d). Analysis of VSV-AV1 replication in malignant primary CLL cells - by viral protein production (Figure 3e), viral M mRNA synthesis (Figure 3f) and virus titer (Figure 3g) - demonstrated that VSV-AV1 replicates in malignant CLL cells and that EM20-25 did not significantly alter viral replication. Together these data demonstrate that VSV-AV1 in combination with EM20-25 synergistically overcomes apoptosis resistance in primary malignant CLL cells without increasing VSV-AV1 replication.

VSV-AV1 in combination with EM20-25 induces apoptosis through the intrinsic mitochondrial pathway.

To further corroborate the data obtained in primary CLL cells, we sought to identify cell lines that expressed BCL-2 protein at levels similar to CLL. Among 12 B-lymphoma cell lines tested, Karpas-422 and Granta-519 naturally overexpress BCL-2 to levels comparable to *ex vivo* PBMCs from CLL patients (Figure 4a and Figure 6a). As with CLL cells, both Karpas-422 and Granta-519 had similar EM20-25 toxicity levels (IC_{50} values of approximately 20 μM) (Figure 2b). Karpas-422 cells were treated with EM20-25 (10 μM) for 30 min and then infected with VSV-AV1 at 10 MOI. The combination led to a 3-fold increase in VSV-induced apoptosis when compared to virus alone ($p<0,05$), as demonstrated by Annexin V-APC staining (Figure 4b). By MTT reduction assay, viability of Karpas-422 was reduced 3-fold with the combination, while loss of viability was only 1.4-fold when Karpas-422 cells were treated with EM20-25 or

VSV alone (Figure 4c). Interestingly, VSV replication was not significantly affected (Figure 4d), indicating that apoptosis observed with VSV-AV1/EM20-25 was not accompanied by increased VSV replication.

To link BCL-2 overexpression with resistance to VSV-induced apoptosis, a Jurkat T cell line expressing BCL-2 under the control of doxycycline (DOX), was used [293]. In this cell line model, a 4-fold increase in BCL-2 was detected at 24 h post-DOX treatment (Figure 4e). At 48 h after VSV infection, 85% of control Jurkat cells underwent apoptosis, whereas in BCL-2-expressing Jurkat cells VSV-AV1-induced apoptosis reached a maximum of 40-50% ($p<0.05$; Figure 4f). Pre-treatment of BCL-2-expressing Jurkat cells with EM20-25 restored VSV-AV1-induced apoptosis to levels similar to those seen in control Jurkat cells (Figure 4f). Confirming our results with Karpas-422 cells, treatment with EM20-25 did not affect VSV replication in Jurkat cells (control or BCL-2 expressing cells) (Figure 4g).

We and others previously reported the importance of the mitochondrial apoptotic pathway in VSV-induced oncolysis [29-30]. Loss of mitochondrial trans-membrane potential is known to trigger cytochrome *c* release from the mitochondria and to promote formation of the apoptosome complex [533-534]. Thus, modulation of mitochondrial membrane potential ($\psi\Delta m$) was examined in Karpas-422 and Jurkat-BCL-2 cells. The combination increased mitochondrial

membrane depolarization (MMP) in both Karpas-422 (Figure 5a) and Jurkat-BCL-2 cells (Figure 5b) but not in control Jurkat cells (Figure 5c), indicating the ability of EM20-25 to enhance VSV-AV1-induced mitochondria depolarization. Furthermore, cytochrome *c* release in the cytoplasm of Karpas-422 cells was observed only with combination treatment (Figure 5d). Taken together, our data indicate that inhibition of BCL-2 function using EM20-25 inhibitor augmented VSV-AV1-induced apoptosis through the intrinsic mitochondrial pathway.

Activation of downstream caspases by the combination was assessed in colorimetric caspase activity assays using substrates specific for caspase-3/7 and caspase-9. The combined use of VSV-AV1/EM20-25 increased caspase-3/7 and caspase-9 activity 6.3-fold and 4-fold respectively, while either VSV-AV1 or EM20-25 alone induced caspase-3/7 and caspase-9 activity by 2-3 fold and < 2-fold ($p<0.001$), respectively (Figure 5e). Furthermore, caspase-3 cleavage detected by immunoblot was 8-fold higher in cells treated with both VSV-AV1/EM20-25, compared to cells treated with EM20-25 alone, and was 2.5-fold higher than cells treated with VSV-AV1 alone (Figure 5f). Similar results were also obtained using Granta-519 cells (Figures 6b-g).

Combination of VSV-AV1/EM20-25 induces apoptosis by disruption of BCL-2/BAX interaction.

BCL-2 inhibits the mitochondrial apoptotic pathway by preventing oligomerization of the proapoptotic proteins BAX and BAK at the mitochondria membrane, which in turn blocks cytochrome *c* release, apoptosome formation and cleavage of caspase-3 [55, 304, 535]. Thus, disruption of BCL-2 family interactions is an important step in triggering mitochondrial apoptosis. As shown in Figure 7, combination treatment was able to disrupt BCL-2/BAX interaction in Karpas-422 cells (Figures 7a,b). Co-immunoprecipitation of BCL-2 following by immunoblot with anti-BAX antibody demonstrated the loss of association between BCL-2 and BAX (Figures 7a,b). An essential step in BAX activation is a conformational change which exposes an epitope at the N terminus of BAX that is occluded in its inactive state [536]. To test whether the combination of VSV-AV1/EM20-25 directly activated the BAX conformational change, cellular protein extracts prepared after 36 h treatment were immunoprecipitated with a monoclonal anti-BAX antibody (6A7) that recognizes the epitope exposed during BAX activation. As shown in Figure 7b, only cells treated with VSV-AV1/EM20-25 display the pro-death conformation, indicating that the combination promotes conformational changes in BAX that are associated with apoptosis. Importantly, the combination did not significantly alter protein expression of other antiapoptotic BCL-2 family members or the expression of BAX (Figure 7c), suggesting that modulation of BCL-2 levels was not involved in

apoptosis induction by the combination. In contrast, EM20-25 alone at 10 μM did not disrupt BAX/BCL-2 interactions, indicating that inhibition of BCL-2 alone is not sufficient to induce apoptosis and the additional stress signal generated by VSV infection was required to disrupt BCL-2/BAX interaction and trigger downstream apoptosis.

VSV-AV1 and EM20-25 selectively kills $CD5^+/CD19^+$ CLL cells and spare normal PBMCs.

Chemotherapeutic approaches should selectively target cancer cells while leaving normal tissues intact. To examine the target cell selectivity of VSV-AV1/EM20-25 treatment, the CD5/CD19 compartment of PBMCs from a CLL patient was examined for apoptosis induction by Annexin-V staining. At 96 h post-infection, apoptosis induced by the combination occurred predominantly in the $CD5^+/CD19^+$ population (R1) (Figure 8a), indicating that the combination is leukemic cell specific; furthermore, EM20-25 did not render the total PBMC population sensitive to VSV-AV1 oncolysis. Indeed, PBMCs isolated from healthy donors were not sensitive to VSV-AV1/EM20-25 mediated apoptosis, as measured by Annexin-V staining (Figure 8b). Also, the dose used to treat PBMCs with the combination did not produce significant cytotoxicity in healthy PBMCs (Figure 8c). Importantly, a direct comparison of healthy PBMCs with primary malignant CLL cells showed that the combination increased the therapeutic index (see Methods) of the two agents by 19-fold (Figure 8d) [537-538].

DISCUSSION

In the present study, we evaluated the therapeutic potential of VSV-AV1 and the BCL-2 inhibitor EM20-25 to induce apoptosis in primary chronic lymphocytic leukemia. Primary *ex vivo* malignant CLL cells were largely resistant to VSV-induced oncolysis, whereas the combination of VSV-AV1/EM20-25 induced synergistic killing of primary CLL cells, as well as increased VSV-AV1 oncolysis in Karpas-422 and Granta-519 cell lines, disruption of BCL-2/BAX interaction and downstream apoptotic events as cytochrome *c* release and caspase-3 cleavage. To our knowledge, this is the first study that demonstrates the value of BCL-2 inhibitors in sensitizing CLL cells to oncolytic virus-induced apoptosis. Our findings support the hypothesis that BCL-2 overexpression creates an apoptosis resistant phenotype in CLL and that blockade of BCL-2 activity sensitizes cells to VSV-AV1-indced oncolysis.

The naturally attenuated VSV-AV1 is a selective oncolytic virus that does not cause significant toxicity in normal human cells. VSV selectivity is achieved by exploiting tumor defects that, while providing cancer cells with growth and survival advantages, compromise the normal innate antiviral program, thus affording a cellular environment that facilitates VSV-AV1 replication and cell killing [11, 14, 181, 528]. Although many cell lines are susceptible to viral oncolysis, primary tumors often exhibit significant resistance to oncolytic virus-induced cell death and this fact has resulted in a search for combination

therapeutic strategies that could overcome resistance. In other studies, we demonstrated that VSV-AV1 in combination with histone deacetylase inhibitors (HDIs) enhance oncolytic activity in *ex vivo* human tumor material and tumor xenograft models, but not in normal primary tissue cultures or peripheral blood mononuclear cells [248]. HDIs have also been reported to increase herpes simplex viruses (HSVs) oncolysis *in vivo* in glioblastoma models [539]. In an elegant study aimed at targeting tumor metastasis, Qiao J. *et al* (2008) used purified populations of normal autologous T cells to carry VSV to lymph nodes and other lymphoid organs. This strategy generated not only metastatic eradication but also antitumor immunity at sites where tumor cell killing occurred [245]. Finally, in this present study, we demonstrated that manipulation of the apoptotic pathway can also contribute to increased efficacy of oncolytic virus in CLL. Non-toxic doses of EM20-25 were used to restore the ability of CLL cells to induce the intrinsic mitochondrial pathway in response to a stress signal – in this case VSV-AV1. The BCL-2 inhibitor had a synergistic effect, only in combination with VSV-AV1 and only in leukemic cells, thus indicating a safer chemotherapeutic regimen [45, 49, 51, 512]. Remarkably, the combination selectively killed the $CD5^+/CD19^+$ CLL population, while sparing the normal PBMC population and did not render normal PBMCs susceptible to VSV-induced oncolysis. After VSV-AV1/EM20-25 treatment of Karpas-422, Granta-519 cell lines and *ex vivo* primary CLL cells, all cells were eventually killed following combination treatment. However, we cannot rule out the possibility that some primary CLL

cells may acquire resistance *in vivo*. Emergence of a resistant population is dependent on several factors, including frequency of exposure to virus and drug, fragility of the leukemic cell population and time of exposure to the treatment. Further studies are required to investigate the possible emergence of an unresponsive population.

Mitochondrial membrane permeabilization (MMP) plays a major role in the apoptosis process [366]. During apoptosis, the outer mitochondrial membrane becomes permeable to proteins located in the inter-membrane space, including cytochrome *c*, which supports formation of the apoptosome, activation of initiator caspase-9 and cleavage of the effector caspase-3 and ultimately processing downstream death substrates [366]. Antiapoptotic members of the BCL-2 family such as BCL-2 and BCL-XL inhibit protein release from the mitochondria, whereas proapoptotic members such as BAX and BAK stimulate this release. Interactions among these pro- and antiapoptotic proteins promote a balance that regulates cell fate [540]. VSV induces mitochondrial dependent apoptosis in a caspase-9/Apaf-1 dependent manner, in part through the viral matrix M protein, which blocks host mRNA export from the nucleus [16-17, 30], through interaction with Rae1/mrnp41 nuclear pore complex proteins [17].

Gaddy and Lyles (2005) showed that the inability of the M mutated VSV-AV1 to inhibit host gene expression resulted in the activation of two major initiator

caspases —caspase-8 and caspase-9 - as well as the executioner caspase-3, suggesting that VSV-AV1 can induce apoptosis via the extrinsic pathway [31]. This cross-talk suggests a mechanism by which VSV-AV1 initially signals through the death receptor pathway, but ultimately both wt and VSV-AV1 require the intrinsic mitochondrial pathway to initiate cell death.

BAX represents the convergence point of VSV-mediated cell death, regardless of the VSV strain. Indeed VSV failed to induce caspase-3 cleavage in BAX/BAK knockout cells, demonstrating a pre-requisite for the intrinsic pathway to trigger efficient apoptosis in VSV-infected cells [29]. The antiapoptotic protein BCL-2 disrupts the apoptotic program by binding and sequestering proapoptotic BCL-2 family proteins such as BAX, thus preventing oligomerization, translocation to the mitochondria and further activation of the intrinsic apoptotic pathway [464]. This study provides further of the importance of BCL-2 and the mitochondrial apoptotic pathway in VSV-AV1-induced apoptosis. Mechanistically, current studies are focused on the specific proapoptotic proteins inhibited by BCL-2 in CLL, as well as the initiator signal used by VSV-AV1 to activate the mitochondrial apoptotic pathway in *ex vivo* CLL.

Overexpression of antiapoptotic BCL-related proteins is a characteristic shared by several malignant diseases [397-398], especially lymphoid malignancies [398, 525, 541]. Downregulation of BCL-2 thus represents an important clinical target

in aggressive CLL. A wide array of BCL-2 inhibitors have been synthesized [532], including oligonucleotides such as G3139 that target BCL-2 mRNA [542-543], and small molecules - EM20-25 [52], ABT-737 [46], HA14-1 [47] and GX015-070 [260] - that recognize the surface pocket of BCL-2 or BCL-XL. These molecules disrupt interactions between pro- and antiapoptotic proteins from the BCL-2 family of proteins, leading to tumor regression with single-agent treatment and serve as an important adjuvant in conjunction with conventional therapy [46, 52, 544]. Conceivably, inhibition of BCL-2 in malignant cells in combination with conventional chemotherapeutics could translate to a better response [55]. Our current understanding of the mechanisms by which BCL-2 controls commitment to cell death provides a strong rationale to augment apoptosis for clinical benefit. The sensitization to VSV-AV1 oncolysis achieved in CLL by agents that increase apoptosis thus identifies a promising therapeutic platform for apoptosis-resistant malignancies.

MATERIALS AND METHODS

Patients and PBMCs isolation

PBMCs were obtained from healthy individuals and CLL patients at the Jewish General Hospital, Montreal, Quebec following written, informed consent, in agreement with the Jewish General Hospital and McGill University Research Ethics Committee (REC). Blood Mononuclear cells were isolated by blood centrifugation (400 g at 20°C for 25 min) on a Ficoll-Hypaque density gradient

(GE Healthcare Bio-Sciences Inc - Oakville, ON, Canada). PBMCs were cultured in RPMI 1640 supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS, Wisent, St-Bruno, Quebec, Canada) and 100 U/ml penicillin–streptomycin. PBMCs were cultured at 37°C in a humidified, 5% CO2 incubator.

CLL patients included in our study were selected on the basis of willingness to donate blood. Median age, sex, and absolute lymphocyte counts were typical of CLL patients in general. Patients were not on therapy at the time of analysis. We determined that B-CLL cases with malignant cells positive for both CD5 and CD19 markers would be used in this study [403, 420, 527]. According to "The National Cancer Institute-Sponsored Working Group (NCI-WG)" guidelines for the diagnosis and criteria for response for CLL, as part of the diagnosis of B-CLL >30% of all nucleated cells from the bone marrow must be lymphoid. In agreement, all PBMCs cells (peripheral blood marrow cells) from CLL patients, used in this study, expressed more than 30% malignant B-cells (Table 1).

Cell lines

The human B lymphoma cell lines (Granta-519 and Karpas-422) used in this study were purchased from the German Collection of Microorganisms and Cell Cultures and were grown in RPMI 1640 medium and Dulbecco's modified Eagle's medium (Wisent, St-Bruno, Quebec, Canada), respectively, supplemented with 10% fetal calf serum, penicillin and streptomycin. All cells were maintained at

37°C and 5% CO2. Wild-type and BCL-2-expressing Jurkat T cells were maintained in RPMI 1640 (Wisent, St-Bruno, Quebec, Canada) supplemented with 10% FCS. The Bcl-2-expressing system was previously described and was a gift from Dr. R. Sekaly (University of Montreal, Montreal, Canada) [293].

Virus production, quantification, and infection

Construction of VSV-AV1 and rVSV-Δ51-GFP (termed here VSV-AV1-GFP) were previously described [11]. Viruses stocks were grown in Vero cells (ATCC, Bethesda MD), concentrated from cell-free supernatants and titrated by standard plaque assay. Briefly, confluent monolayers of Vero cells in 6-well plates were infected with 0.1 ml of serially diluted samples; after 1 h at 37 °C, medium was replaced with complete medium containing 0.5% methyl cellulose (Sigma Aldrich – Oakville, ON, Canada) for 48 h. Vero cells were fixed in 4% formaldehyde and stained with crystal violet. Plaques were counted and titers were calculated as PFU per milliliter. Duplicate experiments were performed and the averages of the virus titers calculated. VSV-AV1 was used for experiments with primary cells and for JC-1 incorporation assay. Primary lymphocytes or lymphoma cell lines were infected with VSV-AV1 at MOI 0.1 PFU/cell (Granta-519) and 10 PFU/cell (primary PBMCs and Karpas-422) for 1 h in serum-free media at 37°C. The cells were then incubated with complete medium at 37°C for the indicated times.

Apoptotic response

EM20-25 5-(6-Chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen -10-yl) - pyrimidine-2,4,6-trione (Calbiochem – San Diego, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 1 mM, aliquoted and stored at -20°C. EM20-25 (Calbiochem) was diluted in medium to a concentration of 10 μM. Cells were cultured at a density of $5x10^6$ cells/ml. A fresh aliquot of EM20-25 was thawed for each experiment and drug added to the media at the desired concentrations for 30 min prior infection. After incubation with the inhibitor, cells were infected with VSV-AV1 or rVSV-GFP (Δ51) at the above described MOIs. Mock-infected cells were used as control. After incubation at 37°C in 5% CO2 for 1 hour, complete media containing freshly thawed EM20-25 at 10 μM was added. Cells were incubated for periods of time varying from 6 h to 7 days and then analyzed for apoptosis by flow cytometry, western blot, caspase activity and virus replication by western blot, plaque assay and RT-PCR.

Flow cytometry

Total PBMCs were isolated as indicated. Aliquots (0.5–1.0 x 10^6 cells), from cells treated or not with EM20-25 (10 μM) and infected or not with VSV-AV1 (10 MOI), were washed with PBS and stained with fluorescein isothiocyanate (FITC)-conjugated CD5; phycoerythrin (PE)-conjugated CD19 for 45 min on ice and allophycocyanin (APC)-conjugated Annexin V for 15 min in 1X Annexin V

binding buffer on ice. B-lymphoma cells Karpas-422 and Granta-519 were treated or not with EM20-25 (10 μM) and infected or not with VSV-AV1 (10 and 0,01 MOI); aliquots (0.5–1.0 x 10^6 cells) were washed with PBS and stained with allophycocyanin (APC)-conjugated Annexin V for 15 min in 1X Annexin V binding buffer on ice. The percentage of apoptotic cells was measured in $CD5^+CD19^+$ B-cell population. Flow cytometry ($1x10^4$ cells/measurement) was performed with a FACSCalibur (Becton-Dickinson - Mississauga, ON, Canada) and analyzed with CellQuest software and FCS Express Version 3 (De Novo Software, Los Angeles, CA). All antibodies were purchased from BD Biosciences.

Semi-quantitative RT-PCR

Total RNA was extracted from cells using RNase extraction Kit (Qiagen – Mississauga, ON, Canada) according to the manufacturer's instructions. RT-PCR was performed using 0.3 μg of RNA ressuspended in RNase-free ddH2O and Oligo dT_{12-18} primer (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's conditions. Reverse transcription was performed using Superscript II at 42°C for 1 h. Following the reverse transcription reactions, cDNA samples were brought to 100 μl final volumes of which 5μl was used as template for each PCR reaction with Taq polymerase. The primer sequences used in this study for PCR were: M protein, Forward 5'-GCGAAGGCAGGCCTTATTTG3' and Reverse 5'-

CTTTTTCTCGACAATCAGGCC-3'; PCR fragments were amplified at an annealing temperature of 55°C for 35 cycles. Products were run on a 1% agarose gel and revealed on the Typhoon 9400 phosphoimager (GE Healthcare Bio-Sciences) and quantification was performed using ImageQuant 5.2 software (GE Healthcare Bio-Sciences).

Protein extraction and Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline, and proteins were extracted in ice-cold lysis buffer containing PBS, 0.05% NP40, 0.1% glycerol, 30 mM NaF, 40 mM β-glycerophosphate, 10 mM Na3VO4, and protease inhibitor cocktail (Sigma Aldrich) in 1/1000 dilution. Extracts were kept on ice for 15 min and centrifuged at 10,000 x g for 25 min (4°C), and supernatants were stored at -80°C. Protein concentration was determined with Bio-Rad protein assay reagent (BioRad - Hercules, CA). Mitochondrial versus cytosol fractions of cells were prepared using a Mitochondria Isolation Kit for Cultured Cells (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Protein extracts (30 μg) were resolved using 12-14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Hybond C Super; GE Healthcare Bio-Sciences Inc). Membranes were blocked for 1 h in 5% non-fat dried milk in TBST (Tris Buffered Saline + 0.5% Tween-20). Followed by incubation with any of the following primary antibodies: anti-rabbit VSV (1:5000), anti-cleaved caspase 3

(Cell Signaling - Danvers, MA; 1:2000), anti-rabbit β–actin (Cell Signaling - Danvers, MA; 1:1000) or anti-mouse BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000). The immunocomplexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence according to manufacturer's specification (ECL, GE Healthcare Bio-Sciences Inc). The cytochrome *c* was analyzed with anti-rabbit cytochrome *c* monoclonal antibody or control antibody anti-mouse β-actin (Cell Signaling - Danvers, MA). Western blot quantification was assessed by densitometric analyses of scanned films with the use of the Scion Image 4.0 software.

Co-immunoprecipitation assay

Cells were lysed with 1% Chaps lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps – Fisher BioReagents, ON, Canada] containing protease inhibitors. Total protein (200 μg) was incubated with 2 μg of anti-BCL-2 or 2μg of anti-BAX (clone 6A7) monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in Chaps lysis buffer at 4°C overnight on a rotator. Immunoprecipitates were collected by incubating with 20 μl protein L-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C, followed by centrifugation for 1 min. The pellets were washed three times with Chaps lysis buffer, and beads were boiled in loading buffer and analyzed by Western blotting using the anti-BAX (clone 6A7) monoclonal antibody.

Measurement of mitochondrial potential by JC-1 staining

The $0{,}5x10^6$ cells were cultured in six-well plates. After treatment with EM20-25 and/or VSV-AV1, cells were collected, washed in PBS and ressuspended in media containing JC-1 (JC-1; CBIC2(3) (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide - Molecular Probes-Invitrogen Canada Inc., Ontario, Canada) at final concentration of 1 mM/L and incubated at 37°C for 15 min. After incubation cells were subjected to flow cytometry analysis on a FACSCalibur (Becton-Dickinson – ON, Canada) and analyzed with CellQuest software.

Quantitative measurement of caspase activity

Cells were lysed in buffer containing 50mM HEPES, pH 7.4, 100mM NaCl, 0.1% CHAPS, 0.1mM EDTA and 1mM DTT. The lysates were clarified by centrifugation and the supernatants were used for enzyme assays. Enzymatic reactions were carried out in 2.5X Pipes buffer containing 20μg of protein lysate and 5μM DEVD-AFC or 5μM LEHD-AFC (both from BioMol International, Plymouth Meeting, PA). Fluorescent AFC formation was measured at excitation 390 nm and emission 538 nm for 30 cycles using a BioRad Fluoromark TM (BioRad).

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 International – Billerica, MA). Cells were seeded in 96-well plates at a density of $5x10^5$ cells per well (PBMCs) or 1 x 10^5 (Karpas-422 and Granta-519 cells). For drug combination studies, cells were incubated with or without EM20-25 (10 μg) or infected or not with VSV-AV1 10 MOI where indicated. For IC_{50} assay increasing concentrations of EM20-25 (300 nM to 200 μM) were used. Plates were incubated at 37°C, 5% CO2 for 7 days. Each experimental condition was performed in quadruplicate.

Statistical, synergism and therapeutic index analysis

The statistical analysis was performed using the Student's t-test. The p-values < 0.05 were considered statistically significant. Average values were expressed as mean ± s.d. The following equation was used to determine the combined cytotoxic effects of EM20-25 according to the method of [545]: CI¼[(D)1/(Dx)1]þ[(D)2/(Dx)2]þ(D)1(D)2/(Dx)1(Dx)2, where (D)1 and (D)2 are the doses of treatments 1 and 2 that produce the x effect when used in combination, (Dx)1 and (Dx)2 represent the doses of treatments 1 and 2 that produce the same x effect when used alone. The combination is additive when CI equals 1.0, synergistic when CI is <1.0, and antagonistic when CI is >1.0.

Therapeutic Index is the capacity or propensity of a drug to affect one cell population in preference to others, i.e., the ability of a drug to affect one kind of cell, and produce effects, in doses lower than those required to affect other cells. Therapeutic Index was calculated by measuring LD_{50}/ED_{50}, where L and E represent lethal (for the normal population) and therapeutically effective (for the malignant population) respectively [546]

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FIGURE LEGENDS

Table 1: Characteristics of CLL patients. Comparative data for CLL donors.

Figure 1: Primary CLL cells are resistant to VSV-AV1 induced apoptosis. (a) PBMCs were isolated from CLL patients (CLL1 to CLL6) and from two healthy volunteers. WCE were analyzed for BCL-2 protein expression by immunoblot. (b) VSV-AV1-induced apoptosis in PBMCs (CLL1 to CLL6) isolated from CLL patients. PBMCs from a healthy volunteer were used as BCL-2 expression control. At 96 h post-infection, apoptosis was measured using Annexin-V staining by flow cytometry. Mock infected cells were used as control (white bars). (c) VSV-AV1 replication in PBMCs isolated from CLL patients and from two healthy volunteer were examined by western blot. VSV-AV1 viral protein expression was monitored by western blot analysis with anti-rabbit VSV antibody; L: large protein; G: glycoprotein and N: nucleopcasid. (d-e) PBMCs isolated from a CLL patient (CLL3) were infected with VSV-AV1 at different MOI (10, 30 and 60 MOI). Mock infected cells were used as control. (d) VSV viral protein expression was monitored as above. (e) VSV-AV1-induced apoptosis in PBMCs from CLL patient measured by Annexin-V staining. Mock infected cells were used as control.

Figure 2: EM20-25 toxicity. (a) EM20-25 Structure. b) Primary B-CLL cells, Karpas-422 and Granta-519 cells were treated with EM20-25 continuously for 96 h (cell lines) and 5 days (primary cells) and cell viability was quantified by MTT Assay. Each data point represents the mean of quadruplicate wells.

Figure 3: BCL-2 inhibitor EM20-25 sensitizes *ex vivo* CLL cells to VSV-AV1 oncolysis. Effect of VSV-AV1/EM20-25 in PBMCs isolated from CLL patients. (a) Effect of EM20-25 on VSV-AV1-induced apoptosis. At indicated times post-infection, apoptosis was measured by Annexin-V staining. Result is reported as percentage of viable cells and values represent the mean of seven experiments ± s.d. Non-infected cells were used as control. (b) VSV-AV1/EM20-25 -induced cytotoxicity in PBMCs isolated from CLL patient (CLL7). At 96 h post-infection cell viability was assessed by MTT assay. Result is reported as percentage of viable cells; values represent the mean of quadruplicate experiments ± s.d. *P*-values of < 0.0001and < 0.0002 are indicated by * and ** respectively. (c) In 7 patients (CLL8-11; 13-15), the percentage of apoptotic cells in each condition was quantified by flow cytometry after Annexin-V staining. Each symbol represents the percentage of Annexin-V positive cells for a particular patient. A paired t-test was used for comparisons (p-values of < 0.0001and < 0.001 are indicated by * and ** respectively). (d) Cleavage of caspase-3 in PBMCs isolated from CLL patient (CLL5); a representative of 5 experiments is shown. PBMCs treated with VSV-AV1/EM20-25 and cleaved forms of caspase-3 were detected by western

blot at the indicate times post-infection. (e) PBMCs isolated from a CLL patient were treated with VSV-AV1/EM20-25 and viral replication was measured. VSV-AV1 Large (L) and Glycoprotein (G) proteins were detected by western blot. (f-g) PBMCs isolated from a CLL patient (CLL9) were treated with VSV-AV1/EM20-25 and viral replication was measured. (f) RT-PCR detection of viral Matrix (M) mRNA synthesis and (g) viral titer determined by plaque assay in Vero cells; values represent the means of triplicate experiments ± s.d.

Figure 4: EM20-25 increases VSV-AV1-induced apoptosis in BCL-2 overexpressing cells. (a) Expression of BCL-2 protein. Different B-cell cell lines, primary CLL cells and PBMCs from a healthy donor were examined by immunoblot for BCL-2 expression. (b) Kinetics of VSV-AV1-induced apoptosis in Karpas-422 cell line. At the indicated times post-infection, apoptosis was measured using Annexin-V staining by flow cytometry; values represent the means ± s.d. of triplicate experiments. P-values of < 0.05 are indicated by *. Mock infected cells were used as control. (c) Karpas-422 cells were cultured in the presence of VSV-AV1/EM20-25 for 48 h and viability was assessed by MTT assay. Result is reported as percentage of viable cells; values represent the means ± s.d. of quadruplicate experiments. P-values of < 0.005 and < 0.002 are indicated by * and ** respectively. (d) Effect of EM20-25 on VSV-AV1-GFP replication in Karpas-422 cells. At the indicated times post-infection, cultures were evaluated for viral replication by flow cytometry. The empty bar represents GFP expression

in mock-treated cells and full bar represents VSV-AV1-GFP expression in EM20-25-treated cells. (e) Expression of BCL-2 protein in Jurkat cells. Wild type Jurkat cells and BCL-2-expressing Jurkat cells were treated with doxycycline (DOX) and BCL-2 protein was detected by western blot. (f) VSV-AV1-GFP-induced apoptosis in control Jurkat (WT) and BCL-2-expressing Jurkat cell lines. At 48 h post-infection, apoptosis was measured using Annexin-V staining by flow cytometry; values represent the means ± s.d. of triplicate experiments. P-value of < 0.05 are indicated by *. (g) Effect of EM20-25 on VSV-AV1-GFP replication in control Jurkat (WT) and BCL-2-expressing Jurkat cell lines. At 48 h post-infection, cultures were evaluated for VSV-AV1-GFP replication by flow cytometry.

Figure 5: Combination VSV-AV1/EM20-25 induced apoptosis in a mitochondrial-dependent manner. (a) Flow cytometry analysis of Karpas-422 cells following JC-1 staining showed increase in mitochondrial membrane depolarization after treatment with VSV-AV1/EM20-25. Mock infected cells were used as control. (b) Flow cytometry analysis of BCL-2-expressing Jurkat cells following JC-1 staining showed increase in mitochondrial membrane depolarization after treatment with VSV-AV1/EM20-25. Mock infected cells were used as control. (c) Pre-treatment with EM20-25 has no effect in VSV-AV1-induced JC-1 incorporation in control Jurkat cells (WT). Mock infected cells were used as control. (d) Cytochrome *c* release into the cytosol. Karpas-422 cells were

treated with VSV-AV1/EM20-25. After 36 h post-infection, cells were collected and cytosolic fractions were prepared as described in Materials and methods. Cytochrome *c* was detected by western blotting. (e) VSV-AV1/EM20-25-induced cleavage of caspases in Karpas-422 cell line. Caspase-3/7 and caspase-9 activities were assayed in cell lysates at 36 h post-infection. Values represent means ± s.d. of triplicate experiment. P-value of < 0.001 is indicated by *. (f) Cleavage of caspase-3. Karpas-422 cells were treated with VSV-AV1/EM20-25. Cleaved forms of caspase-3 were detected by western blot after 36 h post-infection.

Figure 6: Resistance of Granta-519 to VSV oncolysis is overcome by EM20-25. (a) Expression of BCL-2 protein in 6 B-lymphoma cell lines compared with *ex vivo* CLL cells (CLL09) and PBMCs isolated from healthy donors. Expression of BCL-2 was analyzed in WCE extracts by western blot with antibody against BCL-2. β-Actin was included as loading control. (b) Expression of VSV-Δ51-GFP virus in infected cells was determined by FACS. Results are presented as percentage of GFP-positive cells in Granta-519. Mock infected cells were used as control. (c) Apoptosis was measured by flow cytometry in Granta-519 cells treated with EM20-25/VSV-Δ51-GFP infection. Results are presented in bars indicating the percentage of Annexin-V positive cells. Mock infected (control) cells or treated with EM20-25 were included as control, *P*-value of < 0.05 is indicated by *. (d) Analysis of cytotoxicity by VSV-AV1/EM20-25 in Granta-519 cells. Results are presented in bars indicating the percentage of cell viability is

shown. *P*-value of < 0.0001 is indicated by *. (b-d) Data are presented as mean ± s.d. (e) Depolarization of MMP in response to VSV-AV1/EM20-25 treatment assessed by flow cytometry using JC-1 staining. Cells were treated with EM20-25/VSV-AV1 prior to JC-1 staining and flow cytometry analysis. Mock infected cells were used as control. (f-g) Granta-519 cells were treated as above. At 36 h post-infection, cells were harvested and the enzymatic activity of caspases-3/7 (f) and -9 (g) proteases was determined. Results are presented as histograms indicating the amount of caspase activity induced compared to the control sample (mock infected cells). *P*-value of < 0.05 is indicated by *. (b-c) Data are presented as mean ± s.d.

Figure 7: Combination of VSV-AV1/EM20-25 induces apoptosis by disruption of BCL-2/BAX interaction. (a) Karpas-422 cells were treated with VSV-AV1 and EM20-25 alone or in combination. After determined times post-infection, cells were lysed and BCL-2 was immunoprecipitated. BAX interaction was revealed by immunoblot using anti-BAX antibody. (b) Karpas-422 cells were cultured in the presence of VSV-AV1/EM20-25 for 36 h. Following treatment, cells were lysed in 1% Chaps lysis buffer and immunoprecipitated with the indicated anti-BCL-2 or anti-BAX (6A7) monoclonal antibodies. Immunoprecipitates were subjected to immunoblotting using the anti-BAX antibody. (c) Cells were treated as above and pro- and anti-apoptotic proteins were detected by immunoblot. Viral replication (L: large protein; G: glycoprotein,

N: nucleopcasid, P: phosphoprotein and M: matrix) and cleavage of caspase-3 were also monitored by western blot analysis. β-Actin was added as loading control.

Figure 8: VSV-AV1 and EM20-25 selectively kills $CD5^+/CD19^+$ CLL cells and spare normal PBMCs.

(a) Primary PBMCs isolated from CLL patient were treated or not with the combination VSV-AV1/EM20-25. At 96 h post-infection, cells were stained for CD5, CD19 and Annexin V and analyzed by flow cytometry. Cells positive for both CD5 and CD19 antigens representing B-CLL (R1) and non CLL cells (R2) were electronically gated and cell death was assessed, in cells treated with VSV-AV1/EM20-25 compared to the non-infected controls, using flow cytometry. Data are presented as percentage of Annexin-V positive cells measured by FACS in the two different populations, R1 and R2. (b) Effect of EM20-25 on VSV-AV1-induced apoptosis in PBMCs isolated from healthy volunteer. At indicated times post-infection apoptosis was measured by Annexin-V staining. A representative of three experiments is shown. Mock infected cells were used as control. (c) VSV-AV1/EM20-25 did not induce cytotoxicity in PBMCs isolated from healthy volunteer. At 7 days post-infection cell viability was assessed by MTT assay. Results are reported as percentage of viable cells ± s.d.; each experiment was performed in quadruplicate. A representative of four experiments is shown. (d) Treatment of primary CLL cells with VSV-AV1/EM20-25 increased the

therapeutic index compared to PBMCs from healthy volunteers. At 7 days post-infection cell viability was assessed by MTT assay. Results are reported as percentage of viable cells ± s.d.; each experiment was performed in quadruplicate.

Table 1. Characteristics of B-CLL patients

Patient	Age	Gender	Diagnosis	WBC x 109/L	CD5+	CD19+	CD5+/CD19+
1	78	F	B-CLL	10,7	87	73	70
2	71	F	B-CLL	94,4	46	79	37
3	54	M	B-CLL	18,3	83	79	72
4	41	M	B-CLL	42,7	78	71	62
5	48	M	B-CLL	42	66	72	52
6	54	M	B-CLL	12,2	61	56	55
7	41	M	B-CLL	46,7	71	37	33
8	46	M	B-CLL	42,9	78	64	63
9	47	F	B-CLL	221	91	87	85
10	71	M	B-CLL	9,6	91	83	79
11	63	F	B-CLL	29	98	68	67
12	84	F	B-CLL	83,3	92	94	87
13	66	F	B-CLL	17,9	91	97	89
14	63	M	B-CLL	21,4	83	91	81
15	62	M	B-CLL	1,4	61	97	58

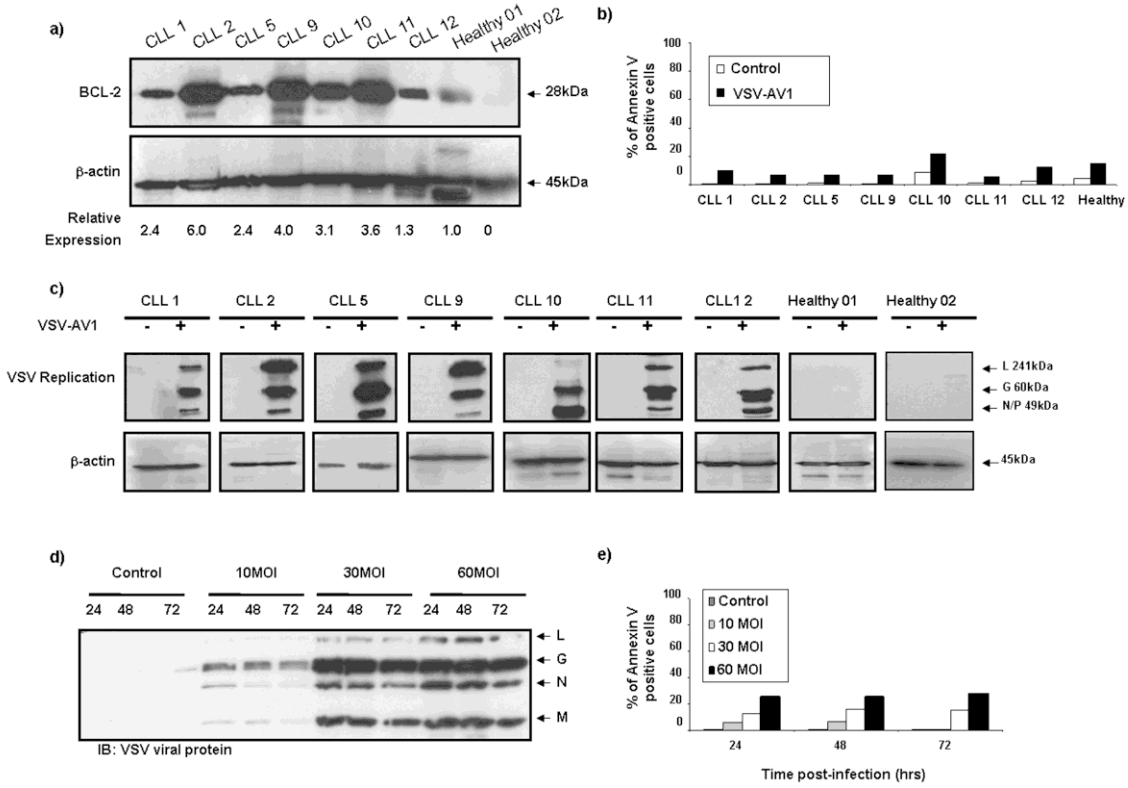
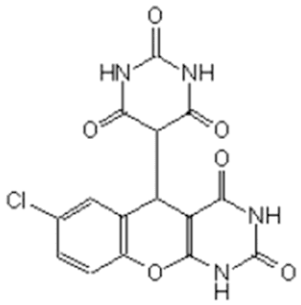

a)
CLL 1
CLL 2
CLL 5
CLL 9
CLL 10
CLL 11
CLL 12
Healthy 01
Healthy 02
BCL-2
28kDa
β-actin
45kDa
Relative Expression
2.4 6.0 2.4 4.0 3.1 3.6 1.3 1.0 0
b)
% of Annexin V positive cells
Control
VSV-AV1
CLL 1 CLL 2 CLL 5 CLL 9 CLL 10 CLL 11 CLL 12 Healthy
c)
VSV-AV1
VSV Replication
β-actin
L 241kDa
G 60kDa
N/P 49kDa
45kDa
d)
Control
10MOI
30MOI
60MOI
24 48 72
L
G
N
M
IB: VSV viral protein
e)
Control
10 MOI
30 MOI
60 MOI
% of Annexin V positive cells
Time post-infection (hrs)

Figure 1

a)


O
HN
NH
O
O
O
Cl
NH
O
N
H
O

b)

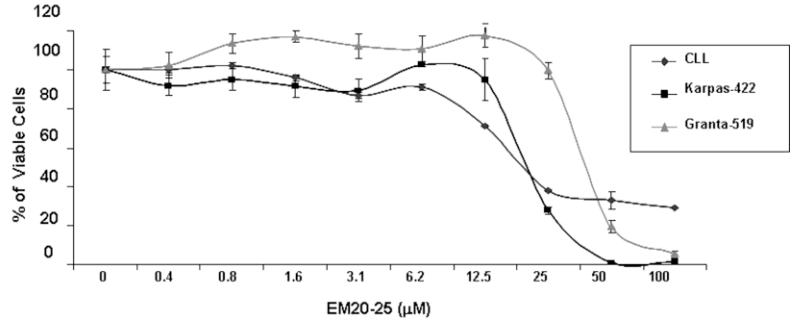

120
100
80
60
40
20
0
% of Viable Cells
0
0.4
0.8
1.6
3.1
6.2
12.5
25
50
100
EM20-25 (μM)
CLL
Karpas-422
Granta-519

Figure 2

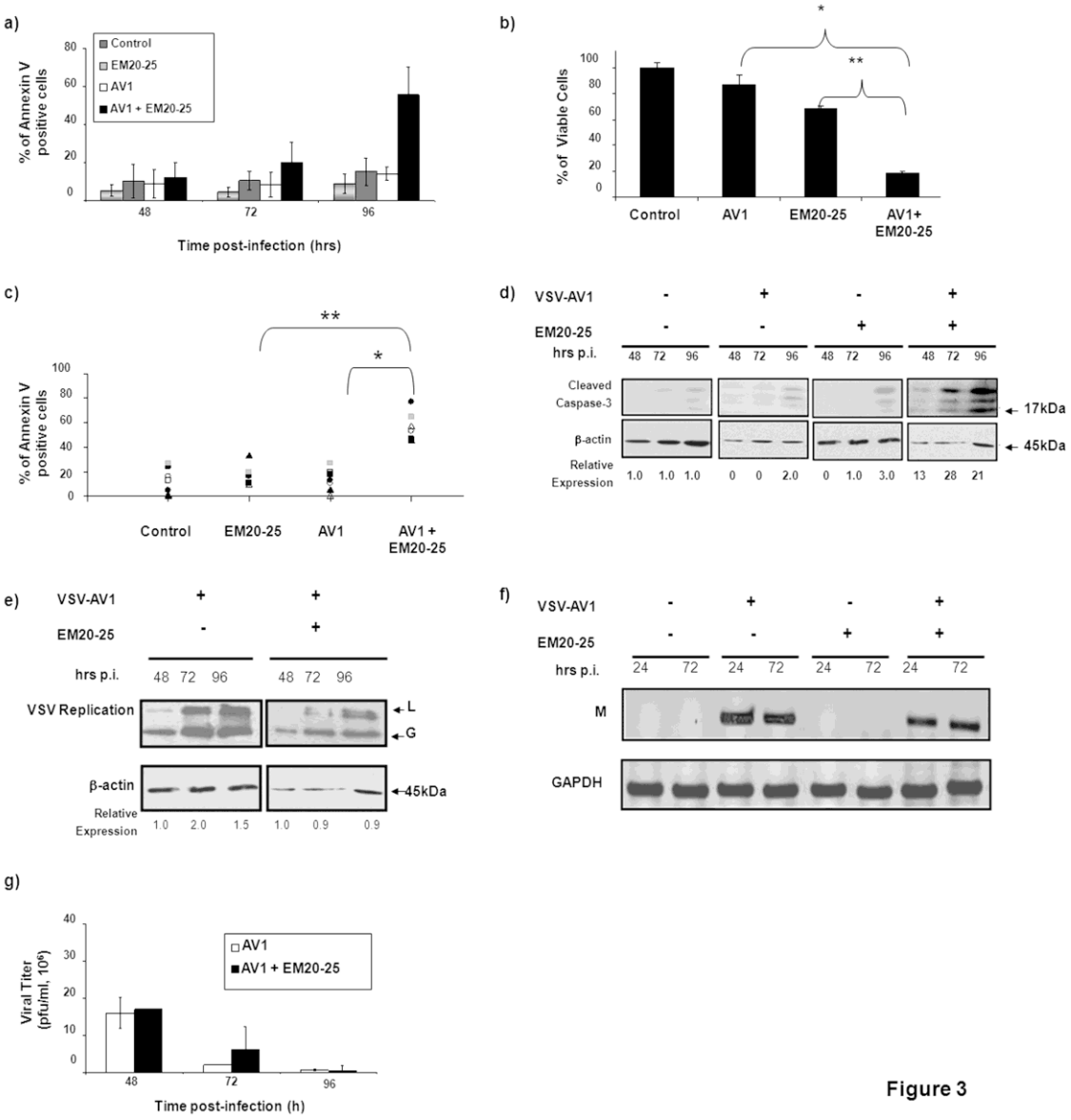

a)
Control
EM20-25
AV1
AV1 + EM20-25
% of Annexin V positive cells
Time post-infection (hrs)
b)
% of Viable Cells
Control
AV1
EM20-25
AV1+ EM20-25
c)
% of Annexin V positive cells
Control
EM20-25
AV1
AV1 + EM20-25
d)
VSV-AV1
EM20-25
hrs p.i.
Cleaved Caspase-3
β-actin
Relative Expression
17kDa
45kDa
e)
VSV-AV1
EM20-25
hrs p.i.
VSV Replication
L
G
β-actin
45kDa
Relative Expression
f)
VSV-AV1
EM20-25
hrs p.i.
M
GAPDH
g)
AV1
AV1 + EM20-25
Viral Titer (pfu/ml, 10⁶)
Time post-infection (h)

Figure 3

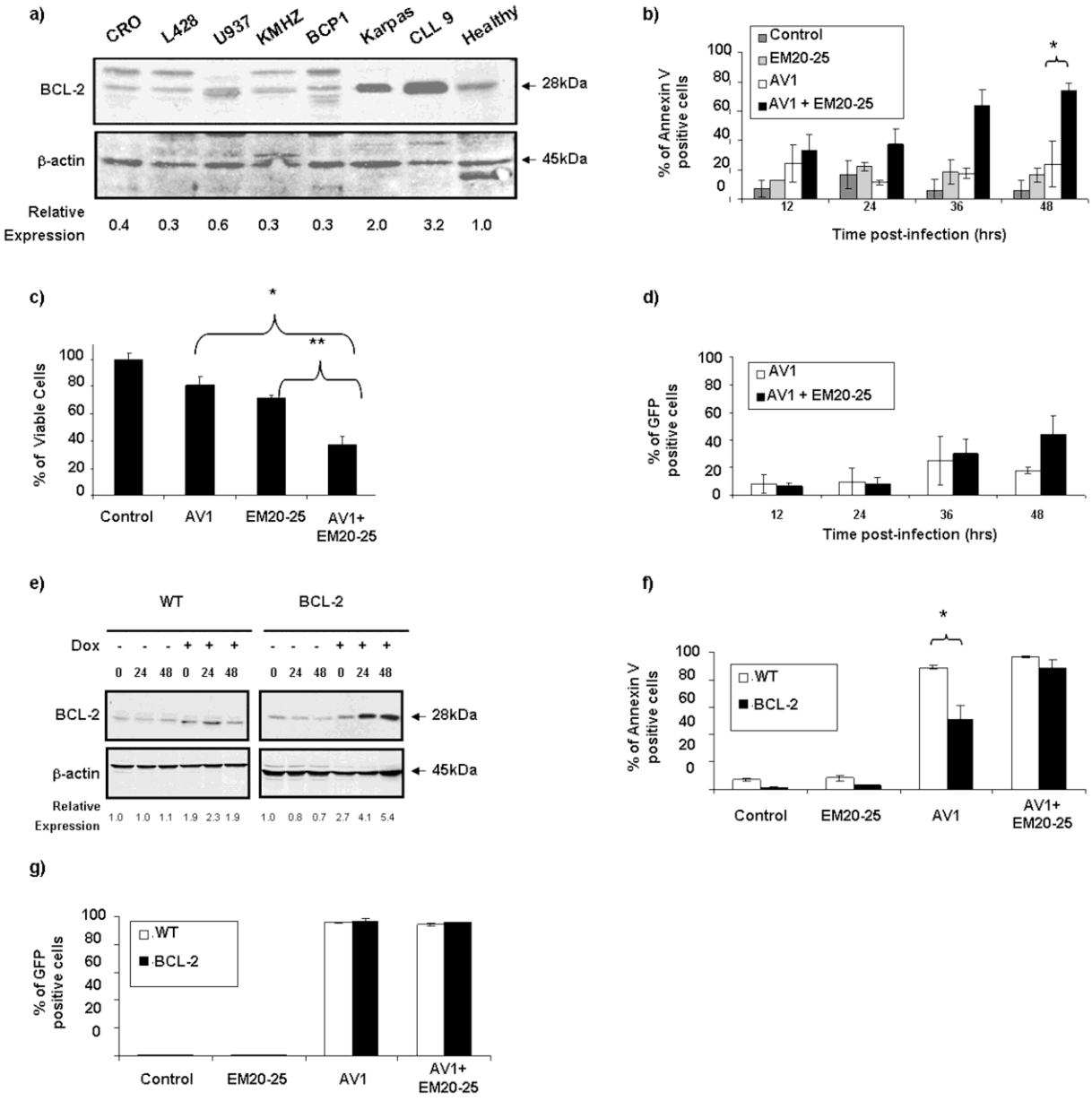

a)
CRO
L428
U937
KMHZ
BCP1
Karpas
CLL 9
Healthy
BCL-2
β-actin
28kDa
45kDa
Relative Expression
0.4 0.3 0.6 0.3 0.3 2.0 3.2 1.0
b)
Control
EM20-25
AV1
AV1 + EM20-25
% of Annexin V positive cells
Time post-infection (hrs)
c)
% of Viable Cells
Control
AV1
EM20-25
AV1+ EM20-25
d)
AV1
AV1 + EM20-25
% of GFP positive cells
Time post-infection (hrs)
e)
WT
BCL-2
Dox
0 24 48 0 24 48 0 24 48 0 24 48
BCL-2
β-actin
28kDa
45kDa
Relative Expression
1.0 1.0 1.1 1.9 2.3 1.9 1.0 0.8 0.7 2.7 4.1 5.4
f)
WT
BCL-2
% of Annexin V positive cells
Control
EM20-25
AV1
AV1+ EM20-25
g)
WT
BCL-2
% of GFP positive cells
Control
EM20-25
AV1
AV1+ EM20-25

Figure 4

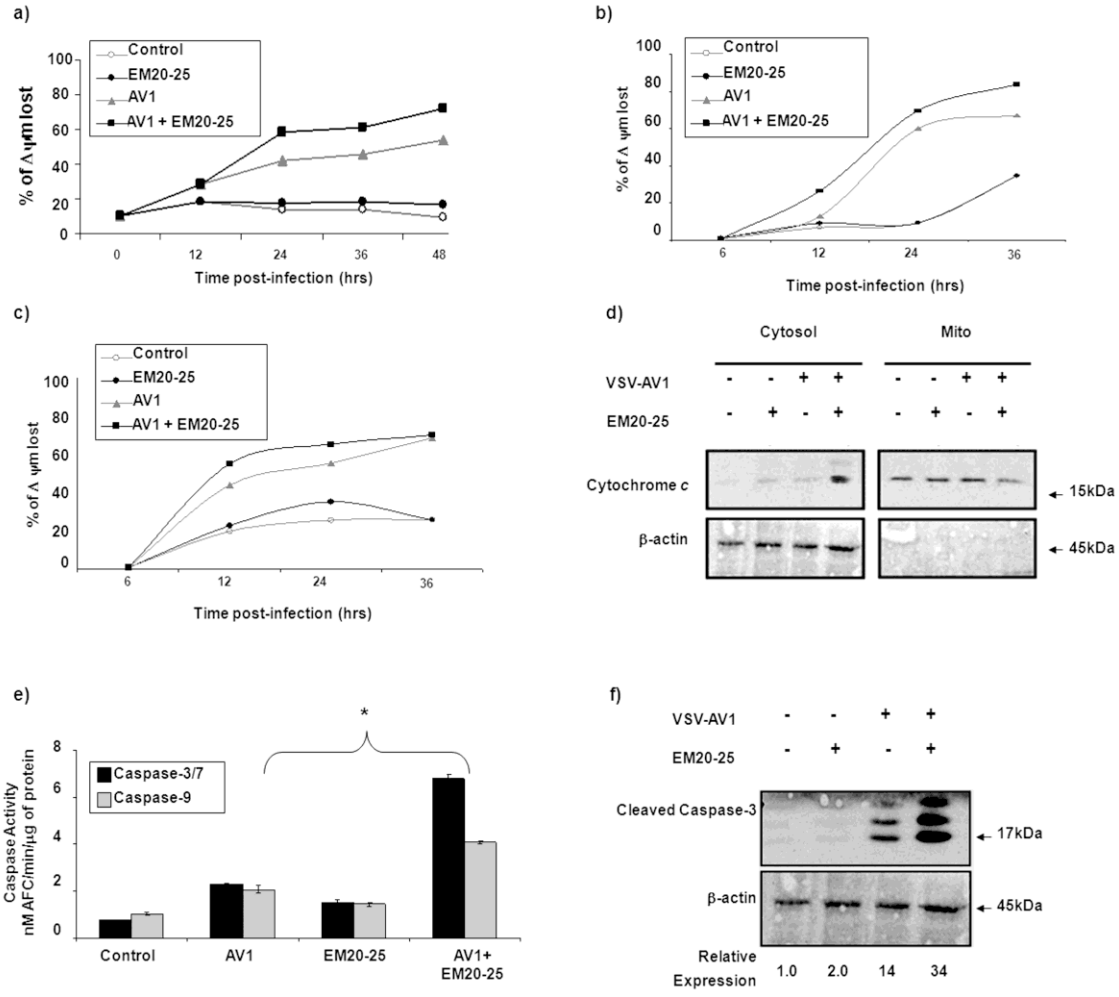

a)
Control
EM20-25
AV1
AV1 + EM20-25
% of Δψm lost
Time post-infection (hrs)
b)
Control
EM20-25
AV1
AV1 + EM20-25
% of Δ ψm lost
Time post-infection (hrs)
c)
Control
EM20-25
AV1
AV1 + EM20-25
% of Δ ψm lost
Time post-infection (hrs)
d)
Cytosol
Mito
VSV-AV1
EM20-25
Cytochrome c
β-actin
15kDa
45kDa
e)
Caspase-3/7
Caspase-9
Caspase Activity
nM AFC/min/μg of protein
Control
AV1
EM20-25
AV1+
EM20-25
f)
VSV-AV1
EM20-25
Cleaved Caspase-3
17kDa
β-actin
45kDa
Relative
Expression
1.0
2.0
14
34

Figure 5

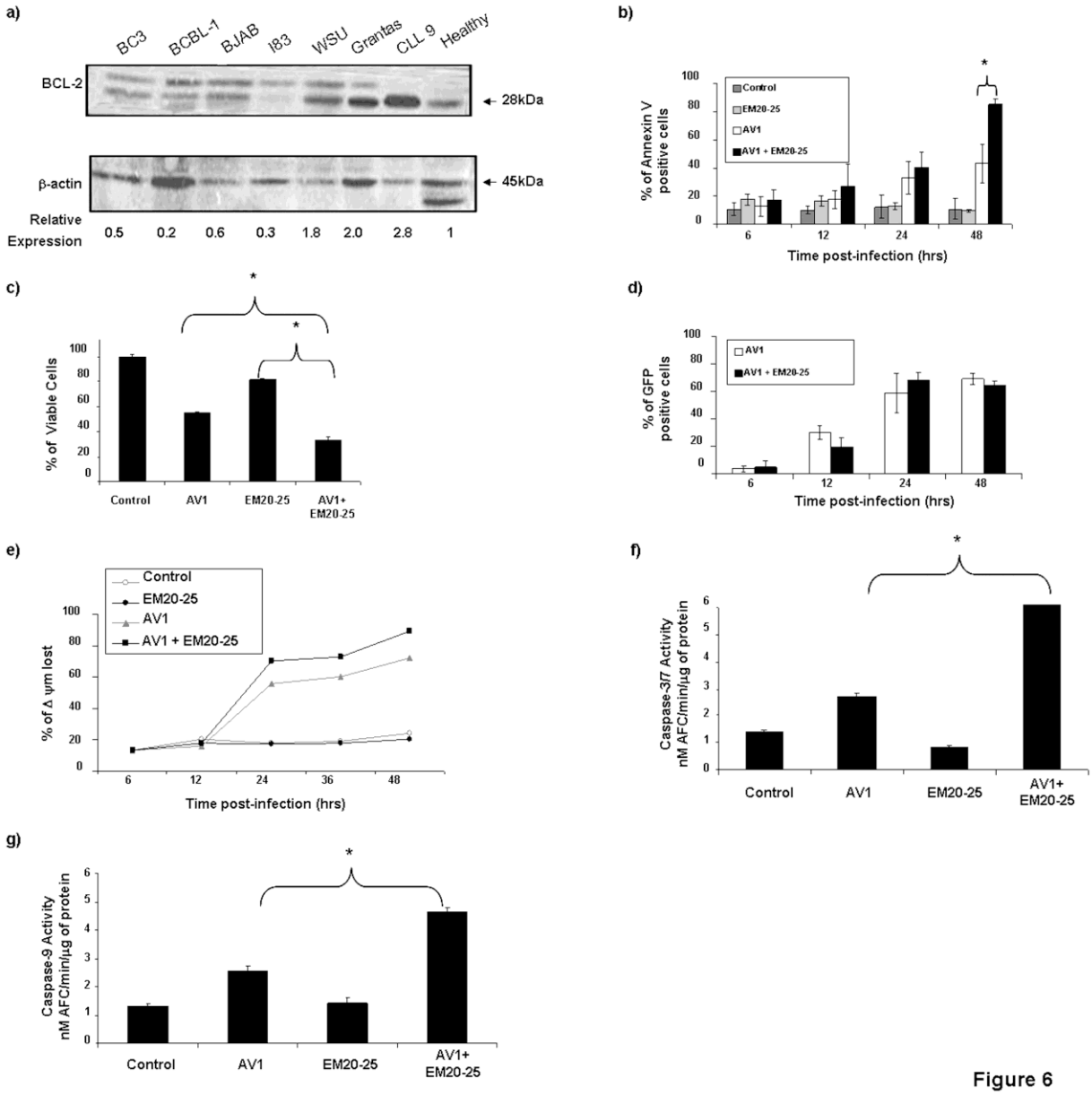

a)
BC3
BCBL-1
BJAB
I83
WSU
Grantas
CLL 9
Healthy
BCL-2
28kDa
β-actin
45kDa
Relative Expression
0.5 0.2 0.6 0.3 1.8 2.0 2.8 1
b)
% of Annexin V positive cells
Control
EM20-25
AV1
AV1 + EM20-25
Time post-infection (hrs)
c)
% of Viable Cells
Control
AV1
EM20-25
AV1+ EM20-25
d)
% of GFP positive cells
AV1
AV1 + EM20-25
Time post-infection (hrs)
e)
% of Δ ψm lost
Control
EM20-25
AV1
AV1 + EM20-25
Time post-infection (hrs)
f)
Caspase-3/7 Activity nM AFC/min/μg of protein
Control
AV1
EM20-25
AV1+ EM20-25
g)
Caspase-9 Activity nM AFC/min/μg of protein
Control
AV1
EM20-25
AV1+ EM20-25

Figure 6

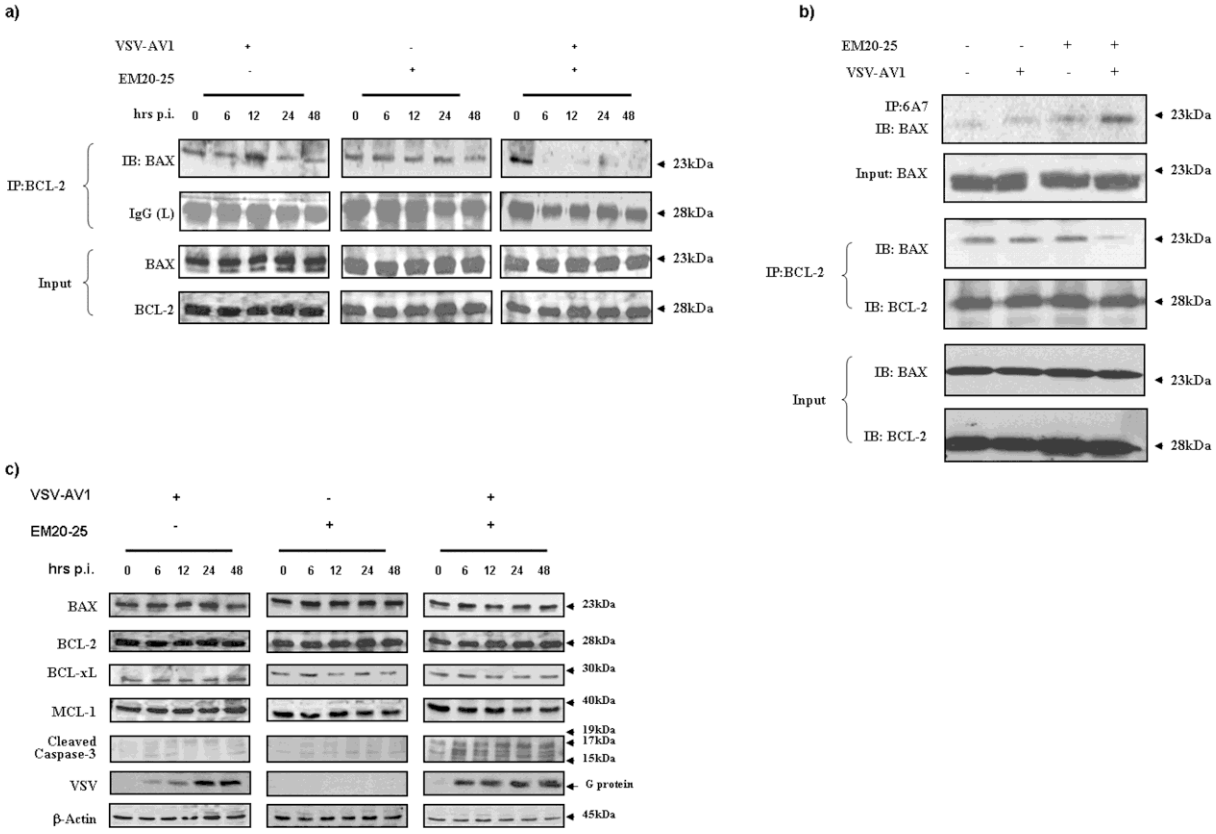

a)
VSV-AV1
EM20-25
hrs p.i.
0 6 12 24 48
IP:BCL-2
IB: BAX
IgG (L)
Input
BAX
BCL-2
23kDa
28kDa
b)
EM20-25
VSV-AV1
IP:6A7
IB: BAX
Input: BAX
IP:BCL-2
IB: BAX
IB: BCL-2
Input
c)
VSV-AV1
EM20-25
hrs p.i.
BAX
BCL-2
BCL-xL
MCL-1
Cleaved Caspase-3
VSV
β-Actin
23kDa
28kDa
30kDa
40kDa
19kDa
17kDa
15kDa
G protein
45kDa

Figure 7

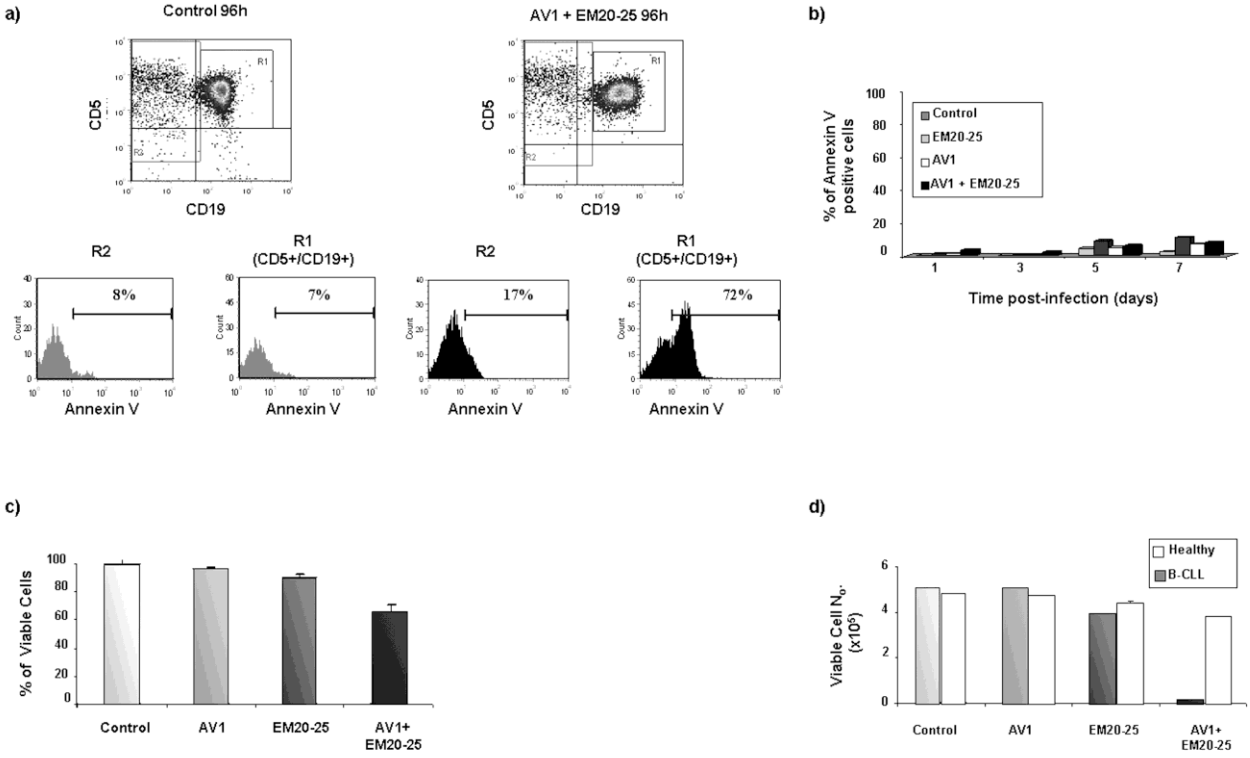

a)
Control 96h
AV1 + EM20-25 96h
CD5
CD19
R1
R2
R2
R1 (CD5+/CD19+)
R2
R1 (CD5+/CD19+)
8%
7%
17%
72%
Count
Annexin V
b)
% of Annexin V positive cells
Control
EM20-25
AV1
AV1 + EM20-25
Time post-infection (days)
c)
% of Viable Cells
Control
AV1
EM20-25
AV1+ EM20-25
d)
Viable Cell No. (x10⁵)
Healthy
B-CLL
Control
AV1
EM20-25
AV1+ EM20-25

Figure 8

CHAPTER 3

VSV oncolysis in combination with the BCL-2 inhibitor obatoclax overcomes apoptosis resistance in chronic lymphocytic leukemia

Preface to Chapter 3

Combinations of small-molecule BCL-2 inhibitors with other chemotherapic agents have been explored to increase antitumor efficacy and the therapeutic index of BCL-2-inhibitors. Our previous study identified that BCL-2 inhibitor is a strong enhancer of VSV-mediated cell death *in vitro*. In this chapter, we sought to further understand the regulatory factors involved in synergism between VSV and small-molecule BCL-2 inhibitors. Collaboration with GeminX provided us with an inhibitor of BCL-2 that is already undergoing clinical trial (Obatoclax). We describe here the *in vivo* anti-cancer properties of the combination of VSV and Obatoclax in tumor-bearing mice. Moreover, we further investigated the molecular mechanism behind the VSV/BCL-2 inhibitor-induced apoptosis.

* This article was published in Mol Ther (2010): Samuel S, Tumilasci VF, Oliere S, Liên-Anh Nguyên T, Shamy A, Bell J, Hiscott J. VSV oncolysis in combination with the BCL-2 inhibitor obatoclax overcomes apoptosis resistance in chronic lymphocytic leukemia. Molecular Therapy 2010, 18:2094-2103.

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 oncolysis of primary CLL cells by vesicular stomatitis virus (VSV). 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* antitumor efficiency of combining the two agents. In combination with VSV, obatoclax induced cell death in primary CLL samples and slowed 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 BCL2-associated X protein (BAX) translocation. Combination treatment triggered the release of BAX from BCL-2 and myeloid cell leukemia-1 (MCL-1) from BCL-2 homologous antagonist/killer (BAK), whereas VSV infection induced Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) expression and increased the formation of a novel BAX/NOXA heterodimer. Finally, NOXA was identified as an important BCL-2 family member involved in VSV-obatoclax-induced apoptosis via knockdown and overexpression of NOXA. Thus small-molecule BCL-2 inhibitors, such as obatoclax, in combination with oncolytic VSV appear to constitute a promising approach to overcoming apoptosis resistance in CLL.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is caused by a defect in apoptosis rather than increased proliferation of $CD5^+$ B lymphocytes [404, 547]. 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) [38, 548]. 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 [466, 549-550]. BCL-2 proteins are subdivided into anti- and proapoptotic classes. Prosurvival members such as BCL-2, BCL-xL, 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 [551-553]. 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 BAK [552, 554] or directly bind and activate BAX/BAK [551].

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 [36, 494]. Encouraging results with BCL-2 inhibitors - either alone or in combination with standard chemotherapies - have been demonstrated

with various cancers, including CLL [36, 514, 516]. 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 BH3 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 pro-apoptotic BCL-2 proteins such as BAX and BAK [521-522]. In preclinical studies, obatoclax has shown cytotoxic efficacy against a variety of cancers including myeloma, breast cancer, mantle cell lymphoma, and non-small cell lung cancer cells [260, 390, 522, 555].

Oncolytic viruses have emerged as a potential treatment of solid tumors and hematological malignancies [28, 39, 45]. By exploiting tumor-specific defects in the interferon (IFN) 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 BH3-only, pro-apoptotic protein NOXA [60-61, 341], or through the extrinsic pathway via caspase-8 and BID cleavage [31, 234].

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

inhibitor [39]. In the present study, we used the pan-BCL-2 family inhibitor obatoclax and we 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 pro-apoptotic 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 IC_{50} of 640 nmol/l, lower than the IC_{50} (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 [552-554]. 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.

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 antitumor effects of VSV-obatoclax were examined *in vivo* in Fox Chase severe combined immunodeficient (SCID) mice-bearing A20 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%).

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 three-fold (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 [556] and as an activator of BID cleavage [557], we suggest 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 [366].

NOXA induction is essential for VSV-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 (IRF-3) and p53-dependent manner [60-61, 341]. 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.

VSV-obatoclax combination triggers BAX translocation and cytochrome *c* release

To further examine 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).

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 [466, 549-550]. 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 [39], 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 might relieve the inhibition to apoptosis.

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, coimmunoprecipitations experiments were performed in Karpas-422 cells. MCL-1 and BAK interacted in non-treated 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 8c, 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 three-fold (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.

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 [64]. Oncolytic virotherapy has emerged as an effective treatment for such cancers [28, 558]. We and various groups have highlighted the ability of VSV to treat disseminated cancers such as CLL [39], adult T-cell leukemia [27] and multiple myeloma [28] 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 [11, 559], 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 [250, 558].

CLL is characterized by overexpression of antiapoptotic proteins such as BCL-2 and MCL-1 [38, 548]. BCL-2 overexpression is a hallmark of this disease and is found in most cases [549]; 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 [466, 549-550]. Under conditions where MCL-1 levels are high, proapoptotic BAK can be sequestered by MCL-1 causing resistance to various therapies [560-561]. 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 [45, 560] but only one group investigated the effect of obatoclax on MCL-1/BAK interactions in CLL [523]; in this case, treatment was used in combination with bortezomib, a proteasome inhibitor that leads to the

accumulation of MCL-1 in CLL cells [562], 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 BAK [561] and this interaction has been characterized in primary CLL following induction of NOXA [560]. Furthermore, NOXA induction has been observed in primary cells from CLL patients following treatment with histone deacetylase inhibitors [560] and aspirin [563] 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 [551, 564]. 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 [551, 565-566]. NOXA is considered a "sensitizer/depressor" BH-3-only protein [551, 564], although a previous report suggested interaction between NOXA and BAX following double-stranded RNA or virus infection [61]. 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 [45, 521-522]. 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 [567]. 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 [60-61, 341] 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 [554]. 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 pro-survival to pro-apoptotic 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).

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 was 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 [39]. 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 [403, 527].

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 collection (Manassas, VA). All cell lines were grown in RPMI 1640 medium (Wisent Inc, St-Bruno, 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 [11]. Virus stock was grown in Vero cells (purchased from ATCC, Bethesda MD), concentrated from cell-free supernatants by centrifugation (15000 rpm/4°C/90 min) and titrated in duplicate by standard plaque assay as previously described [39]. Primary PBMC isolates and Karpas-422 cells were infected with VSV at a MOI of 10 PFU/cell for 1 h 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 International, Billerica, MA). PBMCs were seeded in 96-well plates at a density of $5x10^5$ cells per well. Cell viability was also analyzed by Annexin V and propidium iodide (PI) staining and FACS analysis. For drug combination studies, cells were incubated with or without obatoclax (100 nM) and infected or not with VSV-AV1 (10 MOI) as indicated. To determine the IC_{50}, increasing concentrations of obatoclax (0 to 20 μM) were used. Plates were incubated at 37°C, 5% CO2 and cells analyzed every 24 h 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 [39]. Briefly, cell pellets were lysed in ice cold buffer containing PBS, 0.05% NP40, 0.1% glycerol, 30 mM NaF, 40 mM β-glycerophosphate, 10 mM Na3VO4, and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) in 1/1000 dilution. Extracts were kept on ice for 15 min and centrifuged at 10,000 x g for 25 min (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 (SDS-PAGE) and transferred

to nitrocellulose membrane (Hybond C Super; GE Healthcare Bio-Sciences Inc, Buckinghamshire, United Kingdom). Membranes were blocked for 1 h in 5% non-fat 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; 1:2000); BCL-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA; 1:2000), BAX (Santa Cruz Biotechnology Inc, Santa Cruz, CA; 1μg/ml) and anti-mouse NOXA (Calbiochem, San Diego, CA; 1μg/ml).

Mitochondria isolation

Mitochondria and cytosolic fractions were prepared from Karpas-422 cells after 24h of obatoclax and/or VSV-AV1 treatment, using the Pierce Mitochondria Isolation Kit for Cultured Cells, Reagent-based method according with the manufacturer's protocol. Fractions were analyzed via Western Blot for cytochrome *c* (BD Biosciences, Franklin Lakes, NJ), BAX 6A7, BAX (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and NOXA (Calbiochem, San Diego, CA). 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.

Co-immunoprecipitation of BCL-2 family proteins

2 μg of anti-BCL-2, 2 μg of anti-BAX, 2 μg of anti-MCL-1 monoclonal antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA,), 2ug of anti BAK monoclonal antibody (Millipore, Temecula, CA) or 2ug of anti-NOXA monoclonal antibody (Calbiochem, San Diego, CA) were crosslinked to 20 μg of protein L-agarose beads (Santa Cruz Biotechnology Inc, Santa Cruz, CA) using 0.2M triethanloanime pH 8.0. Cells were lysed with 1% Chaps lysis buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps – Fisher BioReagents – ON, Canada) 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 min. 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 IgG antibody were used as negative controls (data not shown). Protein input (30μg) was run in 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Hybond C Super; GE Healthcare Bio-Sciences Inc, Buckinghamshire, United Kingdom). Membranes were blocked for 1h in 5% non-fat 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 Inc,

Santa Cruz, CA; 1:2000), BAX (Sigma, Saint Louis, MO; 1μg/ml), BAK (Millipore, Temecula and NOXA (Calbiochem, San Diego, CA; 1μg/ml).

RNA extraction and Real-Time PCR

Whole RNA from treated cells was extracted using RNase extraction Kit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. RT-PCR was performed using 1μg and 200ng (for Karpas-422 and *ex vivo* CLL cells respectively) of RNA ressuspended in RNase-free ddH2O and Oligo dT12-18 primer (Invitrogen Canada Inc., Burlington, Canada) according to the manufacturer's conditions. Reverse transcription was performed using Superscript II (Invitrogen Canada Inc., Burlington, Canada) at 42°C for 1h. 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. cDNA 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 RT-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 8 animals per group were used for this study 4-6-week-old female Fox Chase Severe Combined Immunodeficient (SCID) mice (Charles River Laboratories, Pointe Claire, Canada) were injected subcutaneously (sc) with 1×10^6 A20 cells in a 100-µl volume into the hind flanks. Tumors were measured and volume was calculated as [½(length × width^2)] [568]. Once tumors were palpable, animals were randomly assigned to treatment groups and received five intraperitoneal injections of obatoclax (3mg/day/Kg). At days 2 and 5 following obatoclax injection, VSV-AV1 was inoculated intratumorally at 1×10^8 plaque-forming units (PFU) 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 [60]. 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, 1x10^6 Karpas-422 cells were resuspended in 100µl of nucleofector V solution (Nucleofector kit V) containing 100 pmol of doublestranded siRNAs. After electroporation (program T020), 500µl of pre-warmed cultured medium were added to the cuvette, and the cells were

transferred into cultures plates containing pre-warmed culture medium. At the optimal time of gene silencing (24 h post transfection), cells were mock-infected, treated or not with 100 nM of obatoclax or infected with VSV 10 MOI. After 24 h cells were collected, protein extracted and western blot were performed as described.

Transfection of the pcDNA3-NOXA expression vector was carried out as described above with some changes. Briefly, $1x10^6$ 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 h after transfection. 48 h post-transfection cells were collected, protein was extracted and western blot were performed as described.

Statistical analysis

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

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: VSV-obatoclax combination therapy enhances cytotoxicity in $CD5^+$ $CD19^+$ CLL cells. (a)The IC_{50} of obatoclax was determined in PBMCs from CLL patients and healthy volunteers; PBMCs were treated with varying concentrations of obatoclax (0-20 μM) and cell viability was assessed by MTT assay. The results are reported as percentage of viable cells; values represent the mean of quadruplicate experiments ± s.d. (b) Cell viability of PBMCs from 5 CLL patients and 4 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 (100-1000 nM) for 24 h. Cells were lysed in 1% CHAPS lysis buffer and BAX was immunoprecipitated (IP) followed by immunoblotting (IB) with an anti-BCL-2 antibody.

Figure 2: Obatoclax treatment augments VSV-mediated oncolysis in A20 B-lymphoma xenografts in SCID mice. (a) BCL-2 expression. Proteins from 6 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 h post-infection, cell

viability was assessed by MTT assay. Results are reported as percentage of viable cells ± s.d.; 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 intra-peritoneal injections of obatoclax and two intra-tumoral VSV injections, beginning on day 12, through day 16. The star sindicate $P < 0.01$ (*) and $P < 0.001$ (**) comparing tumor size between the single and combination treatment groups. Tumor volumes were calculated as ½(length × width^2) and values are expressed as the mean ± s.d. of tumor volume (n=8).

Figure 3: VSV-obatoclax combination activates the intrinsic apoptotic pathway. The effect of VSV and obatoclax single or combination treatments on cleavage of caspase-3, -8, -9 and BID and NOXA expression in (**a**) PMBCs isolated from CLL patients and (b) Karpas-422 cells was analyzed by Western blot. 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 pre-treated 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).

Figure 4: Induction of NOXA expression is necessary and sufficient to synergistically induce apoptosis with obatoclax. (a-b) Karpas-422 B-lymphoma cells were transiently transfected with siRNA targeting human NOXA (siNOXA) or the nontargeting control pool (siControl). At 24 h post-transfection, cells were treated with obatoclax followed or not by VSV infection. (a) At 24 h post-infection, 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 grey bars represent cells treated with siControl. The data shown are the mean ± s.d (n = 3). (c-d) Karpas-422 B-lymphoma cells were transiently transfected with human pcDNA3-NOXA or empty vector. At 24 h post-transfection, cells were treated with obatoclax (a). At 24 h post-treatment, cells were lysed and NOXA expression, caspase-3 cleavage and VSV replication were analyzed by immunoblot. G, glycoprotein; N, nucleocapsid. M, matrix. (b) Cell viability was determined by FACS analysis after Annexin V/PI staining. (d) Black bars represent non-treated Karpas-422 cells and grey bars represent cells treated with obatoclax. The data shown are the mean ± s.d (n = 3).

Figure 5: VSV-obatoclax induces BAX translocation to the mitochondria and cytochrome *c* release into the cytoplasm. Following 24 h 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.

Figure 6: 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 ± s.d.

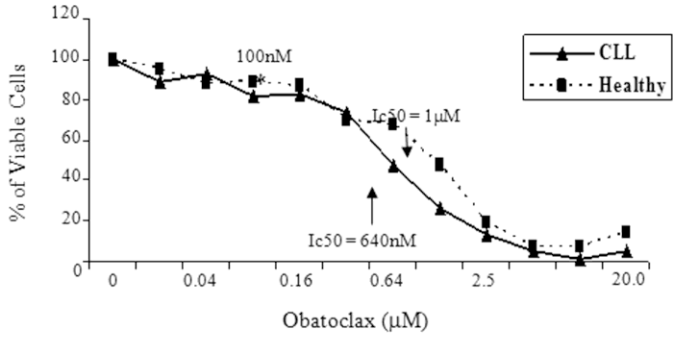
Figure 7: Combination treatment disrupts BCL-2/BAX and MCL-1/BAK interactions and promotes NOXA/BAX heterodimer formation. (a-e) Karpas-422 cells were treated with obatoclax (100nM) and VSV (10MOI) for 24h. Protein lysates were prepared using 1% CHAPS buffer. (a) MCL-1 was immunoprecipitated (IP) from protein lysate and BAK interaction was revealed by Western blot 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 co-immunoprecipitated 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 antibody. MCL-1 immunoprecipitation was performed and bound fractions were analyzed by Western blot for NOXA protein. Input proteins for NOXA and MCL-1 are shown as separate bands at the bottom of the panel. (d) BAX protein was immunoprecipitated, and co-immunoprecipitated NOXA protein was detected by Western Blot using a specific antibody for NOXA. Input proteins for NOXA and BAX are shown as separate bands at the bottom of the panel. (e) The reverse co-immunoprecipitation was performed in VSV and obatoclax treated cells. NOXA protein was immunoprecipitated with NOXA antibody. Co-immunoprecipitated proteins were detected by immunoblot using specific antibody for BAX. Input

proteins for NOXA and BAX are shown as separate bands at the bottom of the panel.

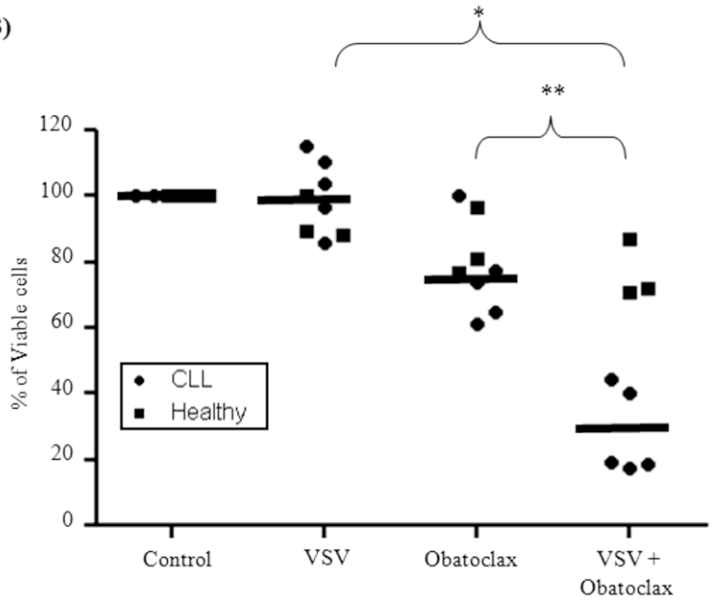
Figure 8: A proposed model of VSV-obatoclax synergism.

In CLL cells, the proapoptotic activity of BAX and BAK are inhibited via association with the antiapoptotic BCL-2 and MCL-1 respectively. Obatoclax occupies the BH3 binding groove of pro-survival 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 BH3-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.

A)


120
100
80
60
40
20
0
% of Viable Cells
100nM
Ic50 = 1µM
Ic50 = 640nM
CLL
Healthy
0
0.04
0.16
0.64
2.5
20.0
Obatoclax (µM)

B)


*
**
120
100
80
60
40
20
0
% of Viable cells
CLL
Healthy
Control
VSV
Obatoclax
VSV + Obatoclax

C)

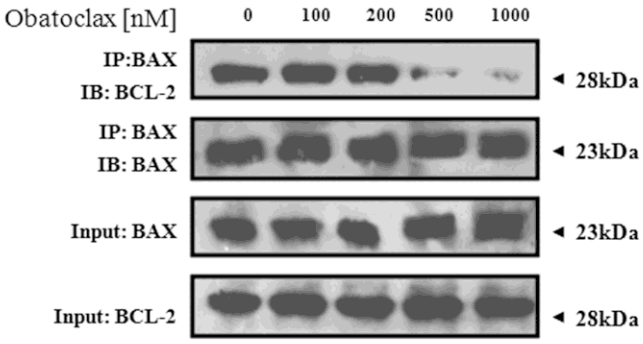
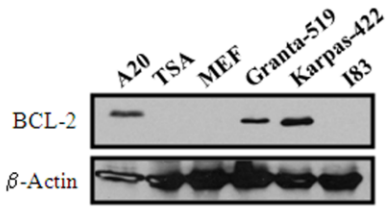
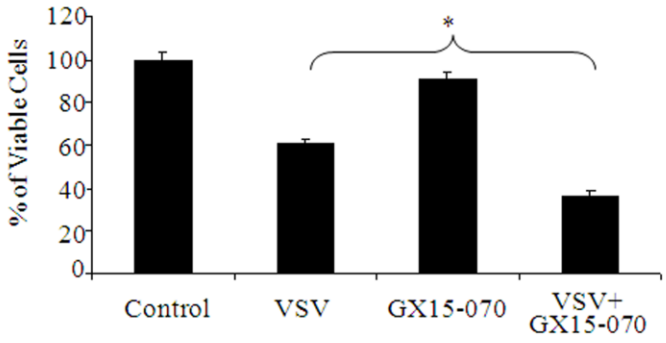

Obatoclax [nM]
0
100
200
500
1000
IP:BAX
IB: BCL-2
28kDa
IP: BAX
IB: BAX
23kDa
Input: BAX
23kDa
Input: BCL-2
28kDa

FIGURE 1

A)


A20
TSA
MEF
Granta-519
Karpas-422
I83
BCL-2
β-Actin

B)


% of Viable Cells
120
100
80
60
40
20
0
*
Control
VSV
GX15-070
VSV+
GX15-070

C)

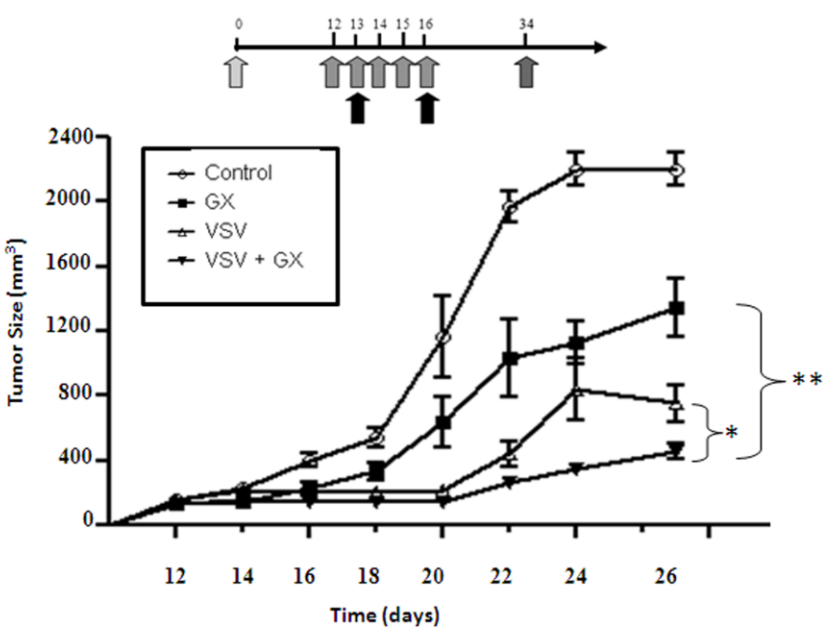

0
12 13 14 15 16
34
Control
GX
VSV
VSV + GX
Tumor Size (mm³)
2400
2000
1600
1200
800
400
0
**
*
12
14
16
18
20
22
24
26
Time (days)

FIGURE 2

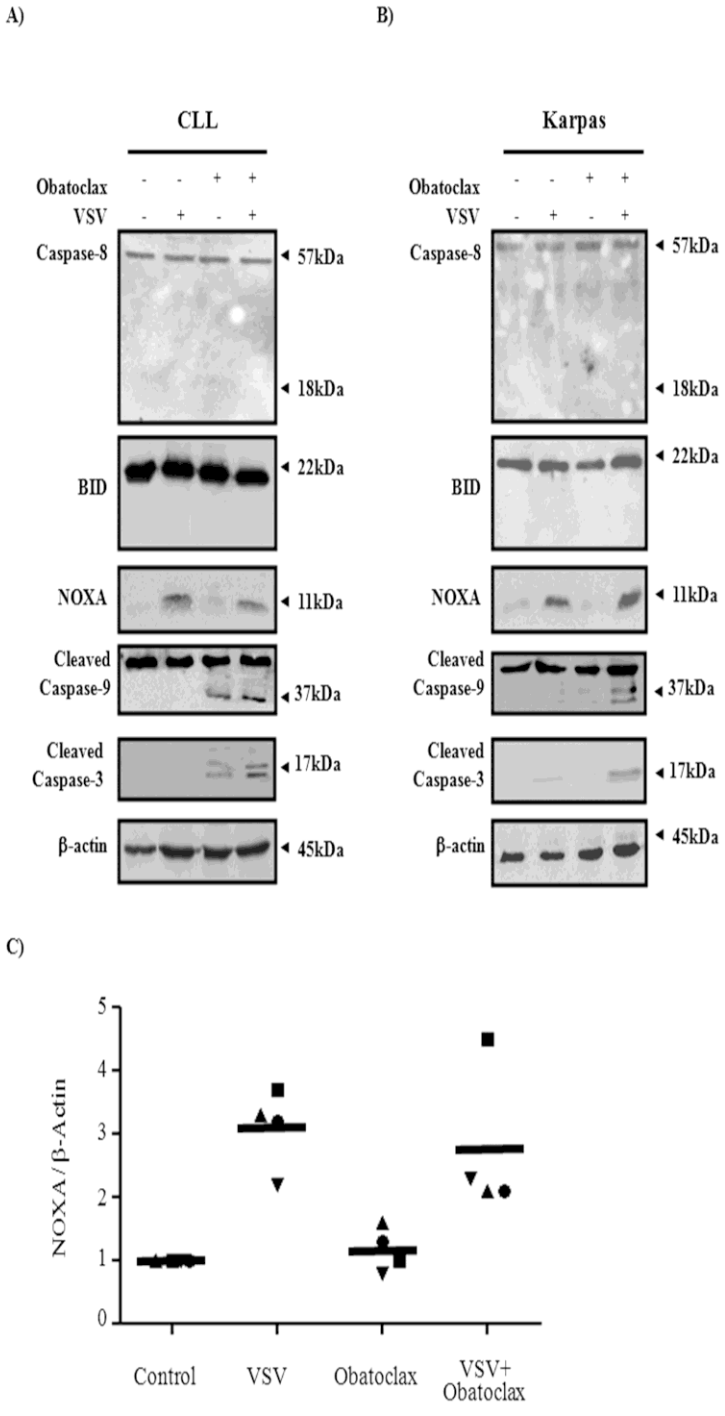

A)
B)
CLL
Karpas
Obatoclax
VSV
Caspase-8
57kDa
18kDa
BID
22kDa
NOXA
11kDa
Cleaved Caspase-9
37kDa
Cleaved Caspase-3
17kDa
β-actin
45kDa
C)
NOXA / β-Actin
0
1
2
3
4
5
Control
VSV
Obatoclax
VSV+ Obatoclax

FIGURE 3

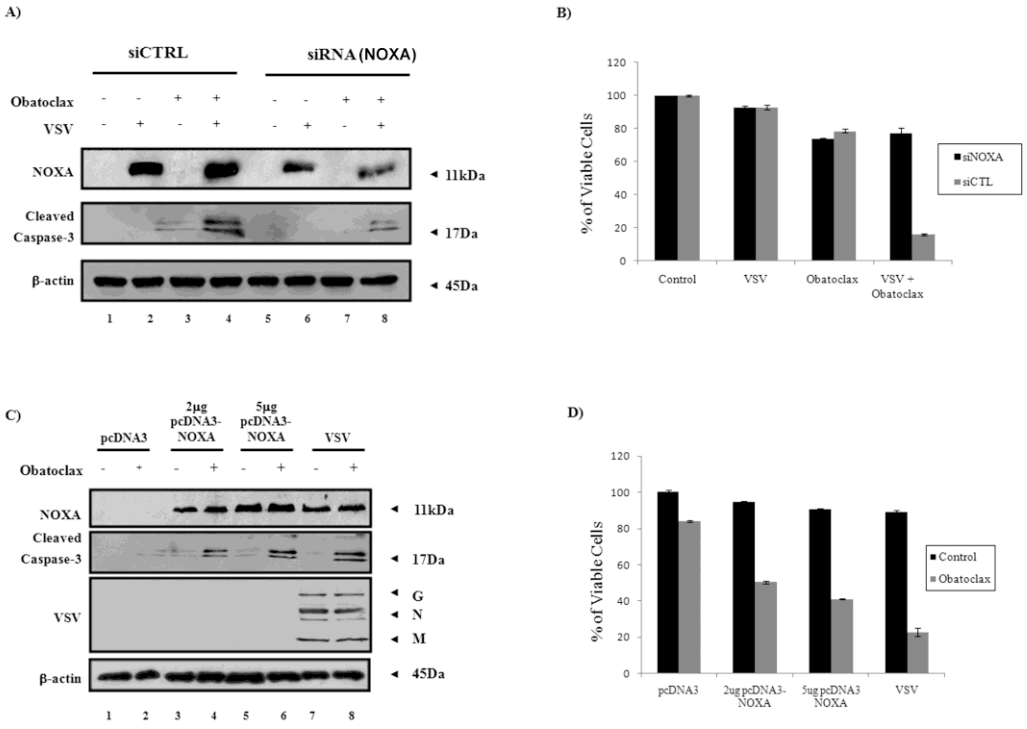

A)
siCTRL
siRNA (NOXA)
Obatoclax
VSV
NOXA
Cleaved Caspase-3
β-actin
11kDa
17Da
45Da
1 2 3 4 5 6 7 8
B)
% of Viable Cells
siNOXA
siCTL
Control
VSV
Obatoclax
VSV + Obatoclax
C)
pcDNA3
2μg pcDNA3-NOXA
5μg pcDNA3-NOXA
VSV
Obatoclax
NOXA
Cleaved Caspase-3
VSV
β-actin
11kDa
17Da
G
N
M
45Da
1 2 3 4 5 6 7 8
D)
% of Viable Cells
Control
Obatoclax
pcDNA3
2ug pcDNA3-NOXA
5ug pcDNA3 NOXA
VSV

FIGURE 4

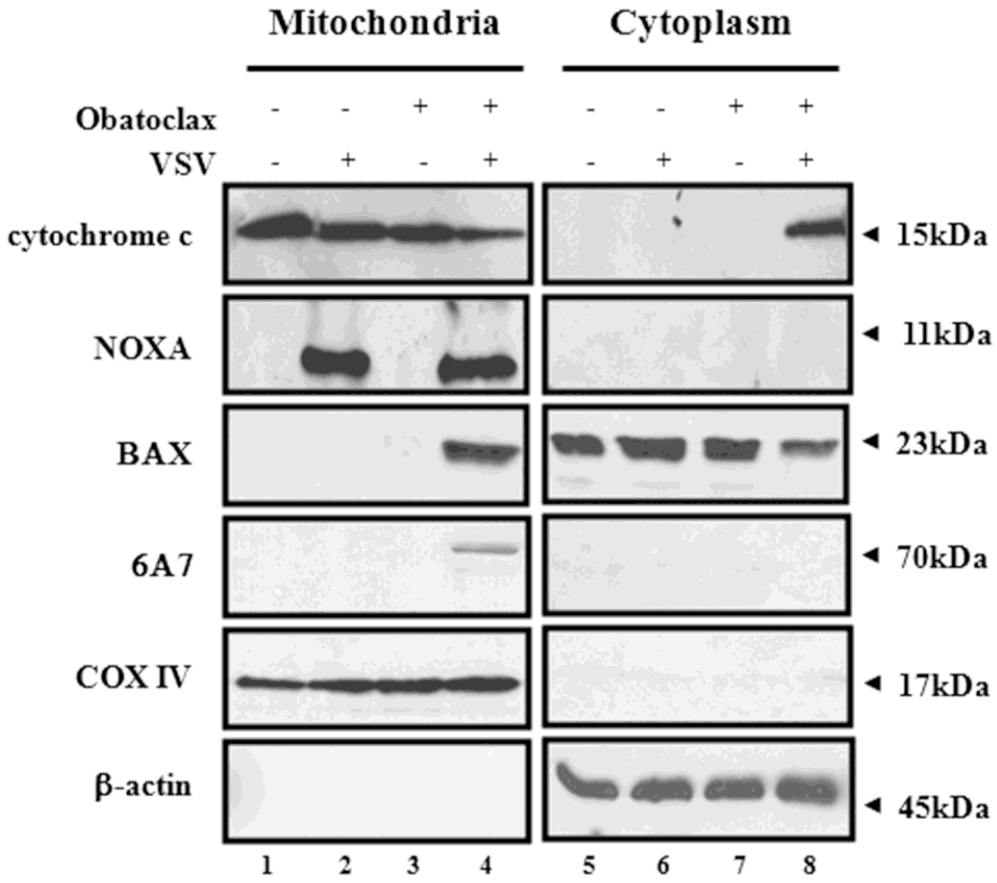
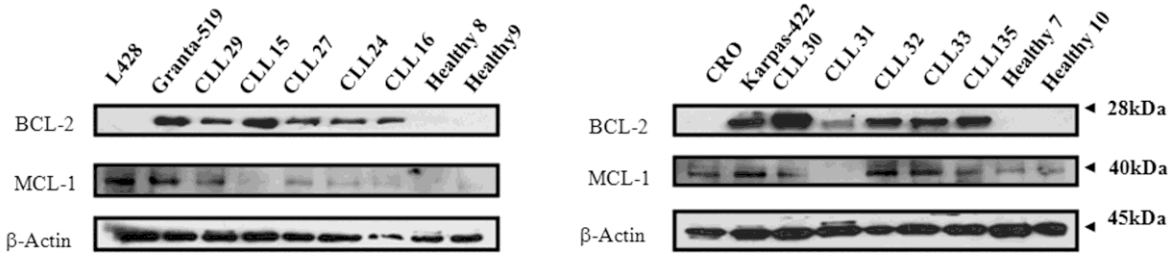

Mitochondria
Cytoplasm
Obatoclax
VSV
cytochrome c
NOXA
BAX
6A7
COX IV
β-actin
15kDa
11kDa
23kDa
70kDa
17kDa
45kDa
1 2 3 4 5 6 7 8

FIGURE 5

A)


L428
Granta-519
CLL29
CLL15
CLL27
CLL24
CLL16
Healthy 8
Healthy9
BCL-2
MCL-1
β-Actin
CRO
Karpas-422
CLL30
CLL31
CLL32
CLL33
CLL135
Healthy 7
Healthy 10
BCL-2
MCL-1
β-Actin
28kDa
40kDa
45kDa

B)

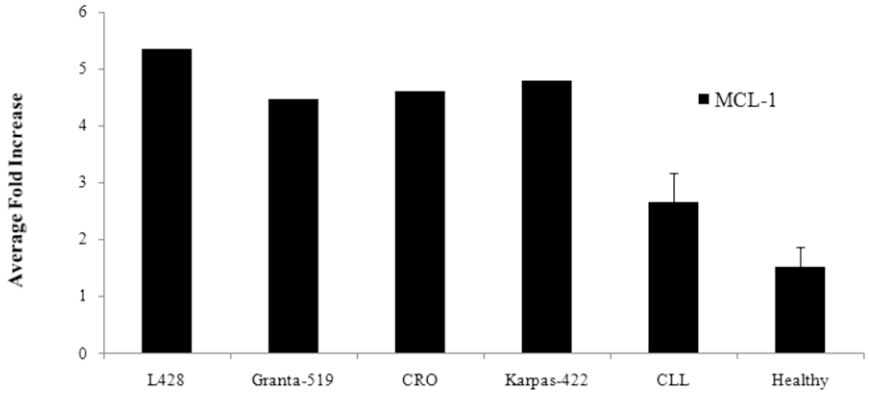

Average Fold Increase
0
1
2
3
4
5
6
MCL-1
L428
Granta-519
CRO
Karpas-422
CLL
Healthy

FIGURE 6

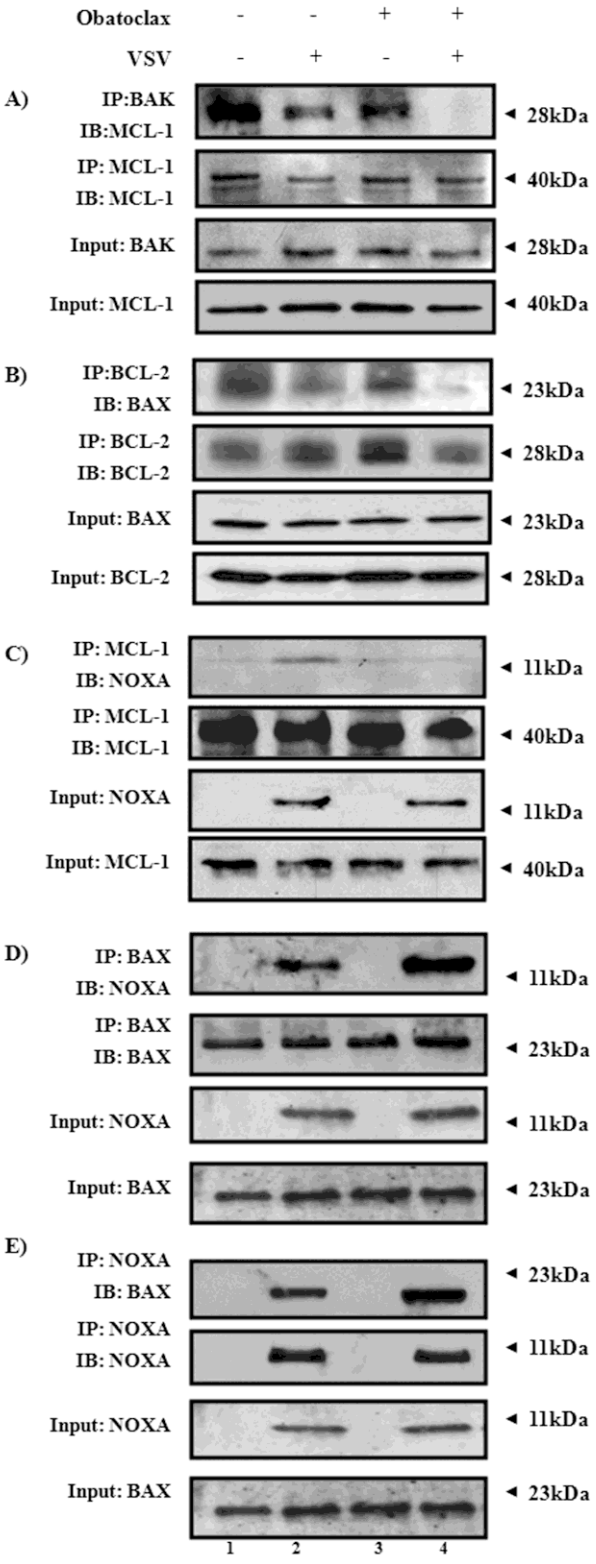

Obatoclax - - + +
VSV - + - +
A)
IP:BAK
IB:MCL-1
28kDa
IP: MCL-1
IB: MCL-1
40kDa
Input: BAK
28kDa
Input: MCL-1
40kDa
B)
IP:BCL-2
IB: BAX
23kDa
IP: BCL-2
IB: BCL-2
28kDa
Input: BAX
23kDa
Input: BCL-2
28kDa
C)
IP: MCL-1
IB: NOXA
11kDa
IP: MCL-1
IB: MCL-1
40kDa
Input: NOXA
11kDa
Input: MCL-1
40kDa
D)
IP: BAX
IB: NOXA
11kDa
IP: BAX
IB: BAX
23kDa
Input: NOXA
11kDa
Input: BAX
23kDa
E)
IP: NOXA
IB: BAX
23kDa
IP: NOXA
IB: NOXA
11kDa
Input: NOXA
11kDa
Input: BAX
23kDa
1 2 3 4

FIGURE 7

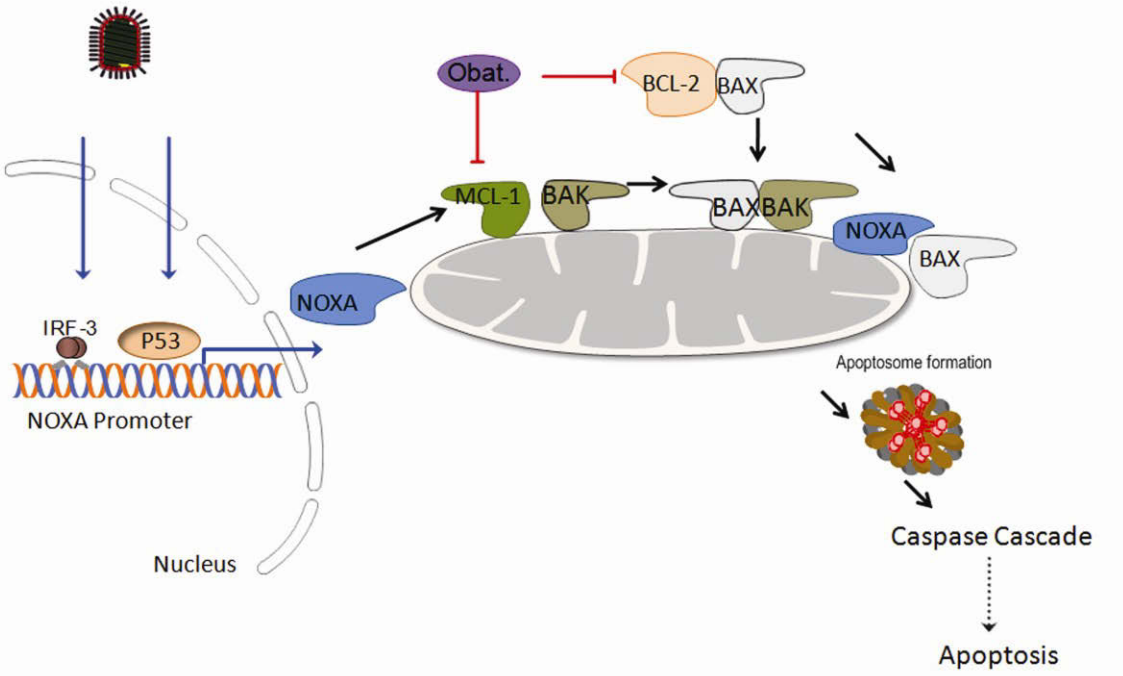

Obat.
BCL-2
BAX
MCL-1
BAK
BAXBAK
NOXA
BAX
NOXA
IRF-3
P53
NOXA Promoter
Nucleus
Apoptosome formation
Caspase Cascade
Apoptosis

FIGURE 8

GENERAL DISCUSSION

Oncolytic viruses, such as VSV, have the remarkable capacity to specifically infect and destroy tumor tissues in animal and human cancer cells, while doing little or no damage to non-cancerous tissues [11, 13, 18, 20, 27-28, 101, 238, 264-265]. Unfortunately, resistance to VSV-induced oncolysis occurs *in vivo* [6, 62-63, 72, 246]. Although VSV is able to replicate and sustain infection within tumor cells, largely due to defects in the interferon pathway, efficient oncolysis by VSV requires induction of apoptosis [11, 18, 20, 60, 234, 378-379]. Blockage of the mitochondrial apoptotic pathway plays a significant role in resistance to VSV virotherapy [63, 245, 247]. Current understanding of the mechanisms of VSV-induced apoptosis allows development of new strategies to overcome resistance to VSV-induced apoptosis in malignancies such as CLL.

Apoptosis is a fundamental cellular process in multicellular organisms. Strict regulation of cell death is essential to the selection and elimination of unnecessary, aging, mutated, or infected cells. Apoptosis also provides a genetically scheduled suicide program that is essential for embryogenesis and homeostasis. When apoptosis is turned on or off unexpectedly, due to genetic mutations, external stimuli, or viral infection, harmful consequences can occur, such as cancer or neurodegenerative diseases [40-44, 295, 303, 384].

The mechanism of the apoptotic pathways is well known. Loss of membrane potential in the mitochondria is one of the earliest events during apoptosis. BCL-2 in the mitochondria inhibits loss of membrane potential, preventing the release of cytochrome *c* into the cytosol and activation of downstream caspases [569-570]. Cell death via the intrinsic pathway is highly dependent on multidomain pro-apoptotic proteins, such as BAX, which permeabilize the outer mitochondrial membrane [55, 354, 356]. BAX is usually expressed constitutively at relatively constant levels, and is mainly regulated post-translationally by other BCL-2 proteins [357-360]. Anti-apoptotic proteins, such as BCL-2, MCL-1, and BCL-xL, bind directly to BAX, inhibit its activity, and prevent it from permeabilizing the outer mitochondrial membrane. The goal of the present study was to determine whether overexpression of BCL-2 and consequent sequestration of BAX, were responsible for resistance of *ex vivo* CLL cells and B-lymphoma cell lines to VSV-induced apoptosis.

Results presented in this dissertation demonstrated that treatment of CLL malignancies with a combination of small molecule BCL-2 inhibitors and VSV virotherapy can induce cell death and tumor regression in *ex vivo* CLL cells and B-lymphomas, such as Karpas-422 and Granta-519. BCL-2 and BAX were shown to exist as a complex in Karpas-422 cells, and the BCL-2 inhibitor, Obatoclax, used as single agent disrupted the BCL-2/BAX complex, but only when used at high concentrations. VSV infection alone did not affect overall expression of

BCL-2, MCL-1, or BAX proteins, induce cell death, or disrupt the BCL-2/BAX complex in CLL cells. Therefore, BCL-2 inhibitors were used in combination with virotherapy to sensitize cells to VSV-induced cell death. Obatoclax or EM20-25 used in combination with VSV disrupted the BCL-2/BAX interaction and activated the pro-apoptotic protein BAX, as evidenced by the appearance of 6A7, the activated form of BAX [536]. Cytochrome *c* was released into the cytosol, caspases-9 and -3 were activated, and cell viability was lost. These results demonstrated that resistance to VSV virotherapy can be reversed by re-establishment of the intrinsic apoptotic pathway.

Results of the present study led to three major conclusions. The first conclusion is that, when VSV-infected CLL cells are treated with small molecule BCL-2 inhibitors, a suboptimal dose of BCL-2 primes the mitochondrial apoptotic pathway to VSV-induced oncolysis. Inhibition of BCL-2 was crucial for VSV-induced cell death, not only in *ex vivo* CLL cells, but also in the B-lymphoma cell lines, Karpas-422 and Granta-519, which naturally overexpress the BCL-2 protein. Jurkat cells that expressed high levels of BCL-2 also presented resistance to VSV-induced apoptosis. Pre-treatment of all of these cell types with BCL-2 inhibitors proved to be effective in overcoming resistance to VSV oncolysis. While the use of BCL-2 inhibitors alone produced only marginal cytotoxicity, pre-treatment of cells with 10 μM of EM20-25 or 100 nM of Obatoclax for 30 min, followed by 10 MOI of VSV, resulted in a significant increase in cell death

in CLL cells (Chapter 2, Figure 3a; Chapter 3, Figure 1b). Cell death promoted by the combination treatment was associated with typical apoptotic characteristics, such as caspase-9 cleavage, caspase-3 activation, and loss of cell viability. These results emphasize the importance of the mitochondrial pathway in VSV-induced apoptosis, despite a requirement for activation of the extrinsic apoptotic pathway in VSV-induced cell death that was demonstrated previous research [234, 369-370].

A lack of suitable animal models for CLL presented a major challenge in linking *in vitro* effects of combined VSV virotherapy and small molecule BCL-2 inhibitors to clinical efficacy. Although a CLL mouse model has been described [571-572] efforts to obtain such animals were unsuccessful. Therefore, *in vivo* data were generated by using murine A20 B-lymphoma cells to produce tumors in SCID mice. A20 cells originated from a BALB/c B cell lymphoma line derived from a spontaneous reticulum cell neoplasm found in an old BALB/cAnN mouse [573]. These cells express high levels of the BCL-2 protein.

Similar to CLL cells, VSV infection alone did not effectively induce cell death in A20 cells *in vivo*, and treatment of affected mice with Obatoclax alone was insufficient to decrease tumor growth. However, as observed in Karpas-422 and CLL cells *ex vivo*, pre-treatment of *in vivo* A20 cells with Obatoclax increased the effect of VSV. Mice treated with either VSV alone or in combination with

Obatoclax exhibited slowed tumor growth than untreated animals, and animals treated with combination therapy had slightly slower tumor growth than mice treated with VSV alone (Chapter 3, Figure 2c). However, slower tumor progression in treated mice did not ultimately result in eradication of the cancer; the tumors grew back in both treatment groups. The partial success of combination therapy in comparison to single treatment underscores the need for further experiments to determine if multiple doses or longer treatment periods could improve the outcome of the combination therapy.

The second conclusion of the research reported in this dissertation is that binding of a small molecule BCL-2 inhibitor to BCL-2 releases BAX protein into the cytoplasm, allowing translocation of BAX protein to the mitochondria. BCL-2 is known to shield cells against apoptosis in response to diverse stimuli, such as ionizing radiation or chemotherapeutic agents [400-401, 574-577]. Results of the present study established that BCL-2 overexpression also confers protection against VSV-induced apoptosis. Induction of apoptosis in leukemias and lymphomas depends on the balance between pro- and anti-apoptotic proteins [40, 43, 464, 578-579]. Failure to activate pro-apoptotic proteins, such as BAX, represents a major barrier to the stimulation of cell death in these cells. Results of the present study showed that the ability of Obatoclax to enhance VSV-induced apoptosis was due to its interaction with BCL-2, which displaces BAX from the BCL-2/BAX complex. The release of BAX allowed its activation and

translocation to the mitochondria (Chapter 2, Figure 5). These results are consistent with previous reports that activation of BAX requires oligomerization, which exposes a hidden epitope recognized by the anti-conformational antibody, 6A7, a marker of BAX activation [362, 536, 580]. Combined treatment with VSV and Obatoclax also triggered the release of cytochrome *c* into the cytoplasm, which is an important marker of the activation of the intrinsic apoptotic pathway.

The third major conclusion of the present study is that the BH3-only protein, NOXA, plays a critical role in VSV/Obatoclax-induced apoptosis. NOXA expression was markedly up-regulated after VSV infection, due to enhanced transcription of NOXA mRNA. This observation agrees with previous studies which found that transcriptional induction of NOXA was involved in virus-mediated apoptosis [60-61, 341, 374]. Furthermore, silencing of NOXA protected Karpas-422 cells against VSV/Obatoclax-induced apoptosis, attenuating the cytotoxic effect of the combination treatment and blocking cleavage of caspase-3. NOXA preferentially binds to anti-apoptotic proteins, particularly MCL-1 [343-344, 561], but might also bind to the pro-apoptotic protein, BAX [61]. Results of the present study showed that NOXA interacted with BAX, following exposure to VSV, and that this interaction was increased in cells that received combination treatment with Obatoclax. NOXA was previously reported to be involved in mitochondrial dysfunction, and NOXA insertion into the mitochondrial membrane was found to result in release of cytochrome *c* [581-582]. Results

presented herein demonstrated that treatment with VSV alone caused substantial upregulation of NOXA in Karpas-422 cells. This upregulation might explain the partial response to VSV observed in these cells, as well as the small difference between VSV and VSV/Obatoclax treatments observed *in vivo*.

Overall, the research presented in this dissertation provides evidence that the intrinsic pathway of apoptosis is highly important for effective VSV virotherapy, especially in malignancies with high expression of anti-apoptotic protein. The results suggest that Obatoclax functionally inactivates BCL-2, causing displacement of BAX. The displaced protein might then bind to VSV-induced NOXA, causing direct activation of the mitochondrial apoptotic pathway.

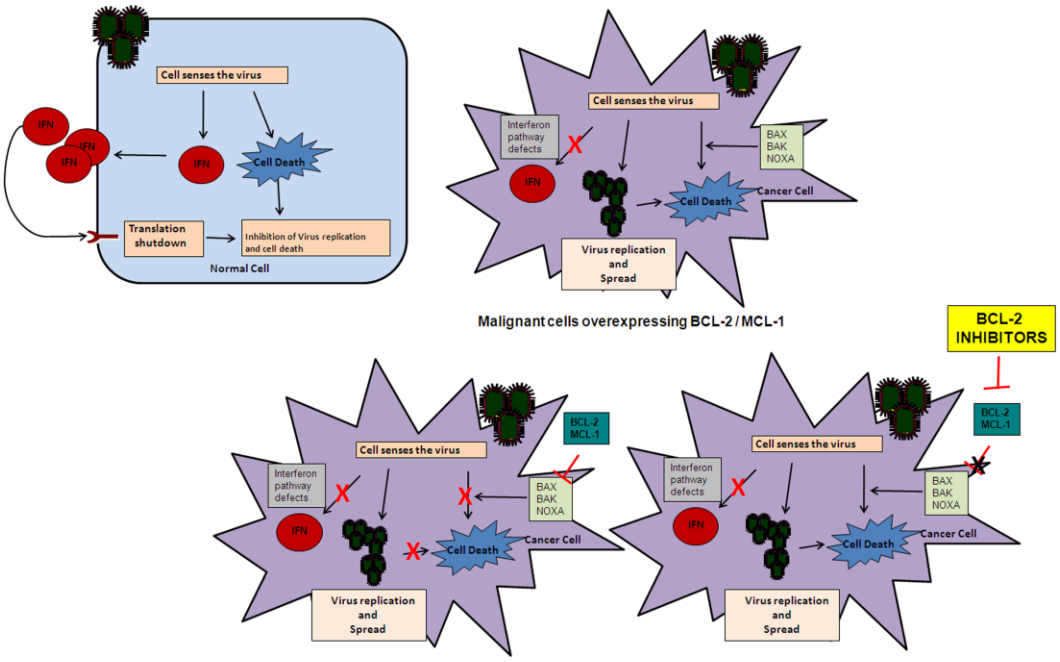


Figure 1. Selective viral oncolysis. In normal cells, VSV infection is controlled by the induction of antiviral cytokines such as type I IFN which block cell and viral transcription thus, inhibiting viral replication. In cancer cells, defects in the IFN antiviral pathway enable VSV to replicate within the cancer cell triggering viral replication and spread and apoptosis as well. Apoptosis induced by VSV in cancer cells is a strong oncolytic mechanism. However, high levels of proapoptotic proteins, such as BCL-2 and MCL-1, in many cancer cells types blocks VSV-induced apoptosis resulting in resistance to VSV oncolysis. The use of BCL-2 inhibitors, such as Obatoclax, overcomes resistance to VSV-induced apoptosis which allows for full activation of the mitochondrial apoptotic pathway by VSV.

CONCLUSIONS AND FUTURE PERSPECTIVES

CLL is a type of small B cell lymphoma for which no effective treatment protocols exist; current treatment is either "wait and see" or monochemotherapy. Success of monochemotherapy for CLL is limited by the development of resistance to the chemotherapeutic agents. Because the connection between cancer and deregulation of apoptosis is strong in CLL, therapeutic strategies that trigger apoptosis in the cancer cells have great potential. The main goal of the present study was to determine the efficacy of small molecule BCL-2 inhibitors in decreasing resistance to VSV-induced virotherapy in CLL cells. The results inarguably established the promise of this novel approach for treatment of malignancies with high levels of anti-apoptotic proteins. The results also raised questions that need to be addressed by future research:

First, a better understanding of the mechanisms of VSV-induced apoptosis in CLL is needed. An endogenous NOXA/BAX interaction is involved in activating the intrinsic apoptotic pathway, but how those proteins trigger apoptosis remains unclear. Moreover, the mechanism that triggers NOXA upregulation by VSV in CLL cells must be determined. Although activation through the p53 pathway has been well studied [343, 347, 352], recent research showed that VSV can induce NOXA-dependent apoptosis in a p53-independent manner [60-61]. To confirm the coordinated actions of NOXA, BAX and IRF-3 or p53 in CLL cells, the ability of p53 and/or IRF-3 to bind to the NOXA promoter should be verified in

primary CLL cells upon VSV infection by EMSA and/or CHIP assays. In addition, BAX/NOXA interaction should be further evaluated by oligomerization assays. Also, BAX/NOXA complexes could be extracted by chromatography for characterization of the complex. Confocal microscopy would help to visualize the co-localization of these two proteins at the mitochondria, upon VSV-infection of CLL cells.

Second, further studies of VSV virotherapy and combined therapies *in vivo* are essential. Information about modulation of BAX, and NOXA expression in mice would elucidate the function of these proteins in VSV infected animals. If the presence of NOXA and BAX is important to the activation of apoptosis, silencing these proteins would obstruct the ability of cells to engage cell death. Knock-out mice for NOXA or BAX and double knock-out would demonstrate if one of these proteins can be activated in VSV--infected mice in the absence of the other protein.

Finally, an animal model of CLL, such as the TCL-1 mice model, is needed to further validate the effectiveness of combination therapy with BCL-2 inhibitors and VSV. The transformed lymphocytes from the TCL-1 mice are G0-1 arrested, clonal, and express $CD19^{+}/CD5^{+}$, as seen in human CLL. Studies performed in these animals would increase significantly the value of the determination of optimal inhibitors, viral doses, administration routes, etc., using imaging and serological studies of cells *in vivo*.

Future research should also investigate MCL-1, another important protein, and its interactions with BH3-only proteins in CLL. The ability of Obatoclax to neutralize MCL-1, in particular, the ability of Obatoclax to displace BAK from its interaction with MCL-1, has been clearly demonstrated [48, 521-522, 579]. The viral strain, VSV-wt, is capable of rapidly eliminating MCL-1, but the matrix protein-mutated strain, AV1, is not [583]. The present study found no MCL-1 degradation in cells infected with AV1 alone or in combination with Obatoclax, but did find disruption of the MCL-1/NOXA interaction. Together, these results strongly suggest that induction of apoptosis by VSV, with or without Obatoclax, depends on cell type. Further research is needed to determine whether MCL-1 plays a role in VSV/Obatoclax-induced apoptosis in CLL cells. As for NOXA/BAX interaction, MCL-1/NOXA and BCL-2/BAX interactions should be further evaluated with silencing of each or both genes in order to determine the importance of each pathway or a possible redundancy of pathways in inhibition of VSV-induced apoptosis in CLL cells.

Virotherapy with VSV has great promise for treating tumors. The relationship between deregulation of apoptosis and resistance to therapy, including virotherapy, is strong, and any therapeutic strategy aimed at triggering apoptosis in cancer cells has potential. Therefore, identification of the apoptotic pathways involved in OV-induced cell death is a key step in the continuing development of

virotherapy. Results of the research presented in this dissertation further our understanding of those pathways and provide a platform for future investigations of the anti-tumor potential of combination therapies with OVs and small molecule BCL-2 inhibitors. Furthermore, the research described herein presents opportunities for investigating the association between BAX and NOXA, particularly how BAX/NOXA triggers mitochondrial apoptosis.

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