Mass Spectrometry-based Analysis to Investigate the Pharmacokinetics and Proteomic Properties of a Viral Sensitizer

by

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Abstract

Attenuated oncolytic viruses (OVs) are a promising alternative cancer therapy to mainstream methods such as radiotherapy and chemotherapy. OV therapy takes advantage of the defective antiviral response present in most cancer cells however heterogeneity amongst target cells and attenuation of OVs to increase their safety profiles has limited the efficacy of this treatment. Our collaborative research group has developed novel small molecules named viral sensitizers (VSeS) capable of enhancing viral infection and cancer-specific cell death. In this study, liquid chromatography-mass spectrometry (LC-MS) methods were developed to study the pharmacokinetic (PK) metabolic activity of VSe1-28 through *in vitro* time course experiments. Furthermore, glutathione (GSH) was identified as an active target for VSe1-28 and two GSH metabolites were identified *in vitro*. It was found that VSe1-28 has a half-life of 3.90 hrs in lysate and 4.83 hrs in growth media. Parallel to this work, proteomic experiments were conducted to confirm the molecular target and mechanism of action of VSe1-28. VSe1-28 has been suspected to inhibit the nuclear translocation of NF- κ B p65 in viral resistant cancer cells through *in vitro* and *in vivo* VSe1-28 modified protein experiments. An MRM method was developed to monitor the formation of the suspected molecular target of interest and a modified tryptic digestion protocol was developed specifically for our work. These new findings will aid in the improvement VSeS and progress preclinical studies one step closer to clinical use in combination with OVs. Future studies are needed to further address the suspected mechanism of action for VSe1-28 to positively confirm the binding location to p65 protein.

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List of Abbreviations

ABC	Ammonium bicarbonate
AC	Alternating Current
ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
AUP	Area-under-the-peak
CEM	Channel electron multiplier
CID	Collision induced dissociation
CysGly	Cysteinyglycine
DC	Direct current
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EI	Electron impact
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
F.A.	Formic acid
GGT	Gamma-glutamyl transferase
GSH	Glutathione
GSSG	Glutathione disulfide
GSTP1	Glutathione s-transferase (π isozyme)
HPLC	High performance liquid chromatography
HSV-1	Herpes simplex virus type 1
IDA	Independent data acquisition
IFN	Interferon
LC-MS	Liquid chromatography – mass spectrometry
LIT	Linear ion trap
LOD	Limit of detection
LOQ	Limit of quantitation

<i>m/z</i>	mass-to-charge
MALDI	Matrix-assisted laser desorption ionization
MASCOT	Protein identification database
MCP	Microchannel plate
MeOH	Methanol
MOA	Mechanism of action
MRM	Multiple reaction monitoring
NL	Neutral loss
OHRI	Ottawa Hospital Research Institute
OVs	Oncolytic viruses
OVT	Oncolytic virotherapy
PBS	Phosphate-buffered saline
PK	Pharmacokinetics
PMF	Peptide mass fingerprinting
Q-TOF	Hybrid quadrupole time-of-flight
RF	Radiofrequency
RT	Retention time
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	Selective ion monitoring
TBS	Tert-butyldimethylsilyl
TEAB	Tetraethylammonium bromide
Th	Thomson
TIC	Total ion chromatogram
TOF	Time-of-flight
TQMS	Triple quadrupole mass spectrometry
VSe	Viral sensitizer
VSV	Vesicular stomatitis virus
WT	Wild type

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Chapter 1: Introduction

1.1 Cancer

Cancer is characterized by abnormal uncontrollable cell growth and the ability to spread to other tissues and organs¹. As reported by the World Health Organization, cancer is the second leading cause of death globally, or one in six deaths as of 2018². In Canada, cancer also poses a large economic burden on the Canadian health care system where cancer treatments and care costs rose from \$2.9 billion in 2005 to \$7.5 billion in 2018³. The transformation from a normal cell to that of a cancerous one is the result of the accumulation of mutations from either genetic and/or epigenetic causes for example, error in replication of DNA, exposure to carcinogens or free radical damage caused by metabolism⁴.

1.1.1 Hallmarks of cancer

The main characteristic hallmarks of cancer which contribute to tumour growth include immune evasion, resistance to cell death, replicative immortality, evasion of growth suppressors, tissue invasion and metastasis, sustained angiogenesis and, uncontrolled proliferation.⁵ The basis of all cancer therapies take advantage of these hallmarks in an effort to suppress, reduce and destroy cancer cells. While chemotherapy and radiation are considered among the most common forms of cancer therapies, they are not selective and lead to damage and cell death of healthy normal cells. As medicinal research continues to evolve, maximizing the efficacy, safety and quality of life of patients is at the forefront of the minds of modern therapeutic researchers⁶. A multitude of alternative therapies are being investigated including oncolytic virotherapy which has shown promising results as an alternative to common cancer therapies.

1.2 Type 1 Interferon Pathway

The canonical type 1 interferon (IFN) signaling pathway is a complex regulatory system involved in the innate and adaptive human immune response⁷. One of the many responses which occur in the antiviral pathway is a signaling response which occurs via protein secretion to boost antiviral responses of neighbouring cells to prevent the spread of infectious agents. Factors which stimulate activation of the immune response include pathogens, environmental factors, and cell host. IFNs signal to neighbouring cells by binding to IFN α receptors (IFNAR), also described as the activation of the Janus kinase (JAK) and activator of transcription (STAT) pathway which leads to downstream phosphorylation of the transducer 1 and signal activator⁷ (STAT1) and (STAT2)⁸. Upon phosphorylation, STAT1 and STAT2 dimerize and associate with IFN regulatory factor 9 to form a protein complex which translocate into the nucleus. At the end of this signaling cascade, the protein complex upregulates the transcription of hundreds of IFN-stimulated genes (ISGs)^{7,9}. This in turn increases cellular resistance to viral infection including entry, replication and exit¹⁰. The type 1 interferon pathway is the most effective defense mechanism against viral infection and is an important host defense which limits tissue damage and prevents autoimmunity.

1.3 Nuclear Factor – κ B

The activation of the canonical NF- κ B pathway promotes tumour progression, angiogenesis, invasion, and resistance to therapy and is a desirable pathway to inhibit to enhance combination cancer therapies^{11,12}. NF – κ B is a nuclear transcription factor which is an important regulator in inflammatory and immune responses. NF- κ B family is comprised of five protein subunits, p50, p52, p65 (RelA), c-Rel, and Rel-B which form homo- and heterodimers¹¹. The most abundant form of NF – κ B is the p50:p65 heterodimer^{11,12} and is found in an inactive state in the cytoplasm bound to an inhibitory protein complex, I κ B family, comprised of either I κ B- α I κ B- β or, I κ B- ϵ . The inhibitor of the κ B kinase complex (IKK) is made up of two catalytic subunits IKK α and IKK β ¹³. When the IKK complex is activated by tumour-necrosis factor- α (TNF- α), CD40 ligand (CD40L), interleukin-1 (IL-1) or lipopolysaccharide (LPS), the IKK β subunit is activated leading to phosphorylation and ubiquitination of the I κ B proteins. This causes the degradation of the I κ B protein, allowing nuclear translocation of the p65:p50 heterodimer and transcription of immunoregulatory and inflammatory genes^{13,14} as shown in figure 1.1.

Research efforts have been made in interventional strategies for regulating the NF- κ B signaling pathway using drug inhibitors. Unfortunately, inhibitors of the canonical p65: p50 pathway that target upstream proteins have been found to have negative off-target effects on other cellular processes¹⁵ as well as disrupt other key pathways¹⁶. Therefore, targeting downstream proteins would ensure site specific inhibition. The structure of NF- κ B, p65: p50, contains two nondisulfide cysteines, Cys120 and Cys38 which participate in DNA binding and transcriptional activation and is of interest to researchers in inhibiting via direct covalent protein binding^{15,17}.

Figure 1.1 Pathways of the production and response to IFN β obstructed by the covalent interaction of VSe1-28 with p65. Adapted from El-Sayes¹⁸.

There are currently a limited number of compounds in clinical use which have been developed to specifically target and inhibit NF- κ B. Fortunately, there are many compounds in use for other clinical purposes which have demonstrated anti-inflammatory and anti-cancer properties which have been proven to inhibit NF- κ B. Common strategies for interrupting NF- κ B transcription factor and its DNA binding activity is to block the binding interface. Compounds such as sesquiterpene lactones have been identified as an interface inhibitor which disrupt DNA binding. Helenalin, in the family of sesquiterpene

lactones, covalently bind to biological thiols such as Cys38 via hetero-Michael addition yielding covalent adducts^{15,19}.

Of high relevance to our group is dimethyl fumarate (DMF), an anti-inflammatory drug clinically approved for use in treating multiple sclerosis and has been found to have anti-cancer properties¹⁷. DMF has been previously demonstrated in our group in inhibiting nuclear accumulation of NF- κ B²⁰. DMF has an α , β unsaturated carbonyl moiety which lends itself to the understanding of the mechanism of action through a Michael-addition with biologically active thiols as shown in figure 1.2. As DMF has been shown to covalently bind to the reactive Cys38 in the DNA binding region of p65¹⁷ it can be assumed that small molecules developed in our lab with a similar α , β unsaturated carbonyl moiety could have a similar mechanism. For the above reasonings, DMF is an excellent inhibitor to use as a positive control in *in vitro* and *in vivo* studies.

Figure 1.2 Structure of dimethyl fumarate.

1.4 Oncolytic Viruses

Oncolytic virotherapy (OVT) was conceptualized following one of the well-studied characteristics of cancer cells being the lack of a fully functioning antiviral response⁵. It is well known that some viruses have cancer-killing properties, such as measles and chickenpox which have been shown to improve tumour regression and remission^{21,22}. As of 2016, oncolytic immunotherapies continued to gain notoriety where a herpes simplex virus type 1 (HSV-1) had been approved by the FDA for the treatment of advanced melanoma²³. The HSV-1 OVT, Talimogene laherparepvec (T-VEC) is a first-in-class oncolytic virus combined with immune-checkpoint inhibitors and radiotherapy²³ approved as an oncolytic therapeutic. Oncolytic viruses preferentially target cancer cells by taking advantage of the genetic mutation differences between normal and cancer cells. Important differences which are considered would be increased metabolism and replication, as they aid viral replication and reproduction. Due to the complexities of tumour mutations and human tissues it is unlikely that one OVT could be developed to target all cancer types. Advantageously, viruses can be rapidly modified to create recombinant viruses designed specifically to target frequent tumour – specific mutations^{22,24}.

Vesicular stomatitis viruses (VSVs) are an RNA virus from the *Rhabdoviridae* family and are an enveloped virus with a single negative strand of RNA. VSVs are also being considered as novel biotherapeutic agents for cancer treatment²⁵. VSVs are a desirable OV candidate for several factors including its well-studied biology, ability to infect multiple cell lines rapidly with high virus yield, small genome for easy genetic engineering and no pre-existing immunity in humans²⁶. OVs primary mode of inducing cell death involves the OV-mediated cytotoxicity created by the infection and lysis of

cancer cells. Furthermore, OV_s act as immune stimulating agents whereby they induce antitumor immune responses through the release of tumour-associated antigens from the lysed cancer cell.²⁷

VSV_s genome is approximately 11 kb which encode for five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and an RNA-dependent RNA polymerase (L)^{26,28}. The VSV G protein mediates viral attachment and fusion to the host cell's membrane. Once fused, the viral genome is released into the cytosol where the replication process begins. The N protein protects the genome while also acting as a template for transcription by the VSV polymerase complex L and P proteins. The M protein is the VSV_s main infection and propagation mechanism as it binds to the nuclear envelope of cells and inhibits nuclear translocation of RNA, proteins, and host gene expression.

The entire lifecycle of VSV_s occurs in the cytoplasm and has been found to be limited in its efficacy to replicate only in cell lines with defective antiviral pathways. Due to safety concerns and mutagenic possibilities and off-target toxicities of WT VSV_s, clinical use has been reduced, however attenuated oncolytic VSV_s remain a desirable candidate for oncolytic virotherapy. To improve the safety and selectivity of VSV_s, genetically engineered virus', or recombinant VSV_s have been developed.

Genetically engineering viruses can enhance the oncospecificity, a method of accomplishing this would be the deletion of the 51st methionine in the M protein which inhibits the M protein's nucleocytoplasmic activity²⁹. The M – mutant VSV, named VSVΔ51 enhances the selectivity to cancer cells by preventing VSV Δ51 from inhibiting nuclear export of antiviral factors in normal cells. As such, normal cells can continue to

produce antiviral response factors while neighboring cancer cells are infected. A major downfall to this strategy is the genetic heterogeneity of tumour cells which limits the efficacy of VSV Δ 51 as it relies on a completely defective antiviral response. Some cancer cells retain some degree of intact antiviral response and render VSV Δ 51 ineffective as a therapeutic. To circumvent this, combination therapies combine VSV Δ 51 with a drug such as checkpoint inhibitors, radiation therapy, or T-cell therapies and now, viral sensitizers.

1.5 Combination Therapies – Viral Sensitizers

As research continues to advance in the field of cancer therapies, traditional non-selective treatments such as chemotherapy and radiation seem antiquated in comparison to the development of new selective treatments. Although new treatments such as OVTs hold promising idealistic potential for a new wave of medicine, obstacles still remain in delivering an effective, fully functioning therapeutic³⁰.

To enhance oncolytic viral infection as described in the aforementioned combination therapies available currently, our group at the Ottawa Hospital Research Institute (OHRI) conducted a high-throughput screen of a group of small molecule compounds. The screening successfully identified numerous compounds capable of sensitizing resistant cancer cells to OV VSV Δ 51 infection which were reduced to fifteen most active hits. One of the most active structures was determined to be VSe1 and analogues stemming from this scaffold were investigated for enhance stability, toxicity, and half-life (See Appendix A). VSe1 and VSe1-28 as shown in figure 1.3 contain electrophilic properties capable of performing Michael addition like NF- κ B inhibitor DMF and was shown to decrease the antiviral responses and transcriptional response to type I

IFN in cancer cells²⁵. Most importantly to this work, normal cells remained resistant to oncolytic viral infection when infected with VSe1-28.

Figure 1.3 Chemical structure of VSe1 scaffold (a) and VSe1-28 analogue (b).

1.5.1 VSe1 and VSe1-28

VSe1 or 3,4-dichloro-5-phenyl-2,5-dihydrofuran-2-one was subjected to numerous experiments to gain an understanding of its physiochemical properties. VSe1 demonstrated enhanced OV propagation and infection in multiple resistant cancer cell lines *in vitro*, *in vivo* and *ex vivo*, with up to 1000-fold increase in viral titers³¹. VSe1 was shown to inhibit the transcriptional response of type I IFN and activation of the antiviral response similarly to DMF. Upon further physiochemical investigations, a plasma stability study was performed where VSe1 suffered from rapid degradation in mouse serum and 0% remained after 3 hours. Despite rapid degradation, VSe1 was found to enhance viral infection of VSVΔ51 in the most virus-resistant cancer cells. As such, VSe1 and VSVΔ51 were proven to be a successful possible OV combination therapy. To enhance the physiochemical properties of VSe1, structural analogs were derived and their structural- activity based

relationships were examined. The most successful analogues were determined to be well-tolerated in terms of improved stability and lower toxicity profiles and enhanced tumour specific OV replication *in vivo*.

Of the derivatives, VSe1-28 or (H-Pyrrol-2-one,3,4-dichloro-1,5- dihydro-5-hydroxy-1-[2-(4-morpholinyl) ethyl]) showed the most improved toxicity, stability profiles and viral enhancement activity. In comparison to VSe1, plasma stability was 0% after three hours, demonstrating the rapid degradation of the molecule. On the other hand, VSe1-28 provides greatly enhanced plasma stability with 40% remaining after 3 hours. A vast improvement upon the VSe1 scaffold was the reduction of the electrophilicity determined through glutathione (GSH) reactivity, where VSe1 demonstrated a half-life of >5 minutes and VSe1-28 a half-life of 96 minutes respectively^{31,32}. Of great importance, VSe1-28 retained similar activity and potency with reduced toxicity where doses of 100mg/kg were tolerated compared to VSe1 which showed toxicity as low as 10mg/kg³¹. VSe1-28 was demonstrated to be the most significantly enhanced derivative upon VSe1 in all studies and has been advanced as a high clinical candidate for combination therapy with VSVΔ51.

1.5.2 Mechanism of Action of VSe1-28

Similar to DMF, VSe1-28 has been found to reduce the expression of IFN β and other antiviral activity. Unlike DMF, the mechanism of action of VSe1-28 had yet to be determined. It had been hypothesized that VSe1-28 and VSe1 inhibited this process by covalently binding to the NF- κ B transcription factor on p65's reactive Cys38. Our group has recently proven this to be true where VSe1-28 inhibits the nuclear translocation of p65:p50 into the nucleus following VSVΔ51 infection in cancer cells³³. To further confirm

these results, LC-MS has been employed to prove binding to Cys38 via various MS scanning modes.

1.6 GSTP1-1

L-Glutathione (GSH) is a tripeptide³⁴ (L- γ -glutamyl-L-cysteinylglycine) consisting of glutamate, cysteine and glycine where the active component is the thiol group of the cysteine. It is a necessary antioxidant which is conjugated via a catalyzed process by GSTP1-1 enzyme in the human body through phase II metabolic processes³⁵. It is best known for the detoxification of electrophilic compounds, xenobiotics and succeeding elimination of toxic compounds³⁶. The homeostasis of glutathione relies on the replenishment of GSH through the reduction of glutathione disulfide (GSSG) and is present largely in the cytosol³⁷.

Reactive oxygen species (ROS) are generated during oxidative metabolism and as a result to cellular response to xenobiotics, cytokines, and bacterial invasion³⁸. The excess accumulation of ROS is related to oxidative stress and is present in high levels in cancer cells due to their elevated metabolic activity, cellular signaling, increased enzymatic activity, activation of oncogene and mitochondrial dysfunction^{37,38}. Although the increase in ROS is attributed to cell damage and death in normal cells, an excess of ROS in cancer cells leads to ROS-mediated signaling pathways activating pro-oncogenic signaling such as the increase in antioxidants such as GSH to promote cell survival and progression^{38,39}. As GSH plays a large role in cell detoxification, the high levels of GSH in cancer cells is often attributed with drug resistance of anti-tumour agents^{40,41}.

Since GSH will be expected at high levels within cells, it is important to develop a fast-acting viral sensitizer with a long half-life with respect to metabolic activity with GSH. Of the numerous analogues developed following the high-throughput screening of viral sensitizers, VSe1-28 was determined to be a suitable candidate with the second highest half-life of 96 minutes with respect to GSH³¹. As such, GSH adducts are predicted to form in metabolomics work and is an important metabolizing enzyme to these studies.

1.7 Mass Spectrometry

Mass spectrometry has become an invaluable tool in analytical fields within pharmacy, chemistry, medicine, food analysis and, forensics investigations⁴². The technique was invented in the late 1890s by Sir J.J. Thomson who provided the first example of mass spectrometry using photographic plates to separate neon into its two different atomic masses (Neon-20 and Neon-22) by their mass-to-charge ratio (m/z , units called Thomson (Th))^{43,44}. Although mass spectrometry has continued to advance in terms of sophistication of software and instrumentation the functionality has remained relatively constant since its inception. A mass spectrometer is composed of three main components which are involved in the development of a mass spectrum. Those being an ionization source, a mass analyzer, and a detector as seen in figure 1.4.

Figure 1.4 The essential components of a mass spectrometer.

A sample enters the mass spectrometer through the sample inlet to an ionization source, which is then ionized either positively or negatively. The ionized sample is transported via an electric field into the mass analyzer, under high vacuum, where they are analyzed by a detector. This produces a mass spectrum which is a function of abundance vs m/z . The first step of mass spectrometric analysis is ionization and vaporization into the gas phase using an ionization source. Numerous types of ionization techniques are now available and are chosen based on the composition of sample and analyte and are classified as either hard or soft ionization. Hard ionization techniques, aptly named due to the harsh methods applied to induce a charged state on a molecule, lead to thermal decomposition or fragmentation. Electron impact (EI) and chemical ionization (CI) used for small organic molecules and atmospheric pressure chemical ionization (APCI) for small biomolecules are examples of hard ionization techniques.

Soft ionization techniques were developed in the mid-1980s which allowed for the ionization of biomolecules for MS analysis without the destruction of molecular structure. As these techniques did not destroy the molecule, unlike hard ionization techniques, these techniques were termed “soft ionization techniques” and used for the study of large biomolecules, enhancing the fields of proteomics, lipidomics and metabolomics. The two soft ionization techniques include electrospray ionization (ESI) used for nonvolatile analytes and biomolecules and matrix-assisted laser desorption ionization (MALDI) used for the analysis of proteins from cell extracts and also biomolecules⁴⁵. It is important to note that although all these techniques differ in their methods of ionization, they all succeed in producing ions in the gas phase for mass analysis.

In the following work, much of the analysis was performed using ESI which is one of the most widely employed ionization techniques coupled to liquid chromatography – mass spectrometry (LC-MS)⁴⁶. The technique is largely applied to large, nonvolatile biomolecules where the sample is dissolved in a volatile solvent containing an ionizing modifier at low concentration such as formic acid (F.A.) at 0.1-0.3% and introduced to the gas phase at atmospheric pressure before proceeding incrementally to high vacuum. Another advantage of ESI is its ability to create multiply charged ions versus MALDI which is only able to form singly charged species. To facilitate ESI, a stainless-steel capillary with a high voltage is used to help oxidize and ionize via the formation of a Taylor cone shown in figure 1.5.

Figure 1.5 Schematic of electrospray ionization (ESI).

In this work, three different mass spectrometers were used employing two different mass analyzer configurations, quadrupoles, and time-of-flight (TOF) which are in the QSTAR XL and Agilent 6546 mass spectrometers, as well as a linear ion trap (LIT) found in the QTRAP 4000 coupled with quadrupoles.

1.7.1 Hybrid Triple Quadrupole Linear Ion Trap

The QTRAP 4000 (AB Sciex) is an enhanced triple quadrupole mass spectrometer (TQMS) because of the addition of the linear ion trap (LIT) mass analyzer. In general, quadrupole mass analyzers contain a set of four cylindrical rods or hyperbolic in cross-section. The quadrupole sets are connected in a linear fashion and act as either a filter for ions or as a collision cell. To filter or scan for ions a direct-current (DC) voltage and a radiofrequency (RF) are applied creating an oscillating electrostatic field⁴⁷ as shown in figure 1.6 A standard triple quadrupole mass spectrometer instrument contains 3 sets of quadrupoles usually denoted as $Q_1q_2Q_3$ where Q_1 and Q_3 are mass analyzers using RF and DC while the second q_2 only employs RF to transmit ions into the second mass analyzer, Q_3 . The first set of quadrupoles, Q_1 , acts as the filter for ions set by the user's mass range or m/z of interest by adjusting RF and DC. Once the ions reach q_2 , fragmentation analysis is performed under a flow of an inert gas such as nitrogen (or argon) to subject ions to collisions resulting in collision-induced dissociation (CID) then transmitted to Q_3 ⁴⁸.

Figure 1.6 A quadrupole mass filter. The ions travel in through the quadrupoles along the x-axis oscillating in the y-z plane by an applied alternating current to be detected.

Four different scanning modes that a TQMS can operate within by varying the ratios of RF and DC of Q_1 and Q_3 are, precursor ion scan, neutral loss scan, multiple reaction monitoring and product ion scan to selectively transmit and analyze fragments. The first two scanning modes mentioned can be described as screening scans due to their respective operating features. The three various MS/MS modes used in this work are seen in figure 1.7.

The precursor ion scan operates by allowing a selected m/z range of precursor ions, also called parent ions, through Q_1 by ramping RF. All precursors are subjected to CID in q_2 , and only the pre-selected m/z product ion produced from certain precursor ions are allowed through to be analyzed. From this, the resulting mass spectrum will display the precursor ions which produced the characteristic m/z product ion. Precursor ion scans provide better sensitivity as it operates by monitoring characteristic fragment ions and is

typically used as a screening method to determine a group of compounds that could potentially produce a characteristic fragment⁴⁹.

Secondly, neutral loss (NL) scanning operates in a function where a mass range is allowed through Q_1 and are fragmented by CID in q_2 . The difference between precursor ion scanning and NL scanning is in Q_3 where it is offset by the mass of the expected neutral loss (in phase from Q_1). Similar to precursor ion scanning, once the fragment is detected that produces the specific neutral loss, the precursor is displayed in the spectrum which can be used for profiling and compound identification.

Product ion scan is a scanning mode which can be used to determine structural information about a molecule of interest. In this mode, Q_1 is employed to only allow a certain mass of interest through to be fragmented in q_2 and the fragments are analyzed in Q_3 ⁵⁰. With the advancements of software such as MassHunter, structural identity can be predicted from the parent and fragment masses and elucidate a chemical formula⁵¹.

Lastly, selected reaction monitoring (SIM) or multiple reaction monitoring (MRM) is an operating method which employs both Q_1 and Q_3 for enhanced sensitivity of compounds of interest. Only a specific mass(es) is allowed through Q_1 and are fragmented in q_2 . In Q_3 , the highest intensity fragment is selected for quantitative analysis of the parent ion. A second fragment can also be selected for qualitative analysis. This scanning method allows for high sensitivity and selectivity. MRMs can be developed to search for a quantifier and qualifier where the parent is selected in Q_1 and the predicted fragment in Q_3 specific to that compound and monitor its decrease through time trialed samples and injections⁴⁹.

Figure 1.7 The various scanning modes of a triple quadrupole mass analyzer.

Quadrupole mass spectrometers are considered low-resolution instruments ($R \sim 1000$) however coupled to another mass analyzer such as a linear ion trap or orbitrap greatly enhances resolution. A linear ion trap (or 2D ion trap) mass analyzer functions in the same capacity as a quadrupole mass analyzer however it additionally consists of two endcap electrodes capable of ‘trapping’ ions^{52,53}. The first ion trap invented, the 3D ion trap (or Paul Trap), suffers from limitations compared to the newer 2D ion traps or orbitraps due to its smaller ion storage capacity and resulting limited duty cycle. A 2D ion trap has been proven to provide higher storage capacities and injection efficiencies⁵⁴. A major advantage to the hybrid triple quadrupole linear ion trap systems is that Q3 can function as

either the third quadrupole in a typical quadrupole mass spectrometer or as the trap function to provide enhanced sensitivity. The combination of TQMS with LIT provides higher quality data for quantitative and qualitative analysis⁵⁵.

1.7.2 Quadrupole Time-of-Flight Mass Spectrometer

Quadrupole time-of-flight (QTOF) MS has a highly sensitive, wide dynamic range, capable of high resolution of a wide variety of positive and negatively charged ions of interest. TOF sends a packet of ions of various m/z accelerated out of the ion source by the backplate, known as an ion accelerator pulse ((+) voltage), through a drift tube where ions achieve mass-to-charge-dependent velocities⁵⁶. The lighter the ion, the higher the velocity achieved which, in turn, reaches the detector sooner. Although TOF instruments are valuable due to their high sensitivity, variability of the ion's starting point within the ion accelerator will cause an ion to have a different ion path/ time even when they are identical mass-to-charge. To compensate for these discrepancies in starting times an ion optic device, a reflectron, which is a set of electrostatic ring electrodes, is placed at the end of the drift tube to refocus ions back to the original ion path⁵⁷. Not only does the reflectron correct for small difference but also doubles the drift tube length which thereby increases the resolution of the mass spectrometer. TOF mass analyzers, in theory, do not have an upper limit in terms of mass range which suits them for high sensitivity and kinetic reaction experiments⁵⁷. Another limitation in the early conception of TOF mass analyzers was that it was ideally suited for MALDI experiments due to the restrictions imposed by the ion sampling efficiency by the pulsed ion source⁵⁸. As a result, an orthogonal accelerator allows for a continuous ion beam for instruments such as ESI-QTOF systems as seen in

figure 1.8. QTOF-MS has become a suitable analytical method of choice for the analysis of drugs in biological samples due to its increased sensitivity, specificity and ever expanding spectral databases⁵⁹.

Figure 1.8 Main components of a QqTOF mass spectrometer. Adapted from Smith⁵³.

1.8 Ion Detectors

MS detectors translate ion current into a quantifiable electrical signal. A channel electron multiplier (CEM) was used in the QTRAP 4000 and a microchannel plate (MCP) detector in the QStar XL and 6546 QTOF. Channel electron multipliers are ideal for charged particle detection in vacuum instruments such as mass spectrometers. The opening, as shown in figure 1.9, is slanted in a manner that allows for the correct number of electron collisional events with the CEM walls. With each collision more electrons are released, amplifying the cascade and, by the end of the channel, hitting the anode and producing a measurable electrical current⁶⁰.

A microchannel plate is a collection of smaller channel electron multipliers. MCP channels (and CEM) are semiconducting to allow for the replenishment of charge and promote electron ejection (multiplicative characteristics)⁶¹ making each channel a dynode structure. MCP's can be used singly, or in a stacked format, to enhance the current signal output. A stacked format, otherwise known as a chevron, can enhance the electron multiplication factor by $10^4 - 10^7$ ⁶¹. The chevron style MCP detectors are used in TOF mass analyzers due to the varying spatial return of a charged particle returning from the drift tube.

Figure 1.9 The operation of a chevron within a microchannel plate detector. Adapted from Wiza⁶¹.

1.9 Liquid Chromatography – Mass Spectrometry

Although mass spectrometers can separate analytes based on their mass-to-charge, preconcentration and further separation based on other molecular properties can be achieved by coupling MS with high-performance liquid chromatography (HPLC). In this work, reversed-phase (RP) LC was coupled to the mass spectrometer (QTRAP 4000 and Agilent 6546 QTOF) for ESI-MS analysis of small molecules and biological samples. Pharmaceutical compounds are most monitored by RP-LC-MS as they contain hydrophobic moieties which interact with the non-polar C18 column.

1.9.1 Mass Spectrometry – based Metabolomics

Metabolomics is the study of metabolite profiles of a biological system for the purpose of identification and quantification of biological processes^{62,63}. This technique is advantageous due to MS's ability to simultaneously analyze numerous variables *in vivo* in a complex biological sample. This work focused on the pharmacokinetics (PK) properties of a viral sensitizer through the study of its metabolic activity, the third step in the four-step process of absorption, distribution, metabolism and excretion (ADME). The study of metabolomics holds many challenges including an extensive list of metabolites where comprehensive databases are continuously being improved upon. Various methods have been applied based on the topic and sample preparation where for example one cell culture sample could be divided into investigating the lipid metabolites versus the polar metabolites present in the cells⁶³. Furthermore, once metabolites are identified, structural identity is important to PK studies and requires a high resolution instrument to elucidate the metabolite's mass. A strategy for structural determination of metabolites is the

development of labeled tracers or compounds. Of interest to our work, is the synthesis of a multi-atom isotopically substituted VSe1-28 which would shift the mass-to-charge enough to positively identify the metabolites and aid in structural determination.

Metabolites can be monitored via MRM analysis or post data acquisition making the use of either triple quadrupole MS or QTOF amenable for analysis⁶⁴. To accomplish this, samples are treated with the compound of interest and monitored over time to investigate the complex biological changes occurring within the sample. Monitoring the decrease of the compound of interest over time confirms the degradation or metabolic processes occurring, followed by the increase in metabolites. These increases and decreases in peak intensities can be monitored via total ion chromatograms (TIC) and extrapolated MS, MS/MS scans. Once identified, extracted ion chromatograms (EIC) can be used to extract masses of interest for quantification. Advanced software techniques can further extract and perform statistical analysis on spectral matrixes to identify potentially informative changes occurring in complex biological samples.

1.9.2 Mass Spectrometry – based Proteomics

MS-based advancements in the area of protein analysis can be attributed to researchers such as Klaus Biemann who performed one of the first experiments to determine the molecular weight of small molecules for the purpose of determining their structure⁶⁵. With these experiments the study of proteomics began, developing a powerful, effective, and essential tool in the analysis of proteins found in organisms, tissues, or cells. Vast improvements, in terms of separation and peptide sequencing, have led to a booming

field of study capable of quickly analyzing large complex biological samples using sophisticated software and specialized MS proteomics methods.

Amino acid monomers are the building blocks of peptides and proteins. These monomers contain a characteristic amine (-NH_2) and carboxyl group (-COOH) bonded to an α - carbon, R-group, and hydrogen. Once three or more monomers are bonded together it is then called a peptide, which is a short chain amino acid sequence. Although there is no strict definition of when the classification of a peptide becomes a protein it is generally agreed upon that a peptide chain of >50 peptides can be considered a protein⁶⁶. The peptides and proteins can be analyzed by LC-MS using proteomics. The two proteomics strategies used to analyze samples are: top-down or bottom-up⁶⁷. In the top-down method, proteins remain intact and MS/MS is performed to determine the protein's structure, which is comparable to traditional intact molecular analysis. This technique is most commonly used for protein identification and analysis of post-translational modifications⁶⁸. Bottom-up is the most mature and widely used proteomics technique which involves using a protease enzyme to cleave the peptide backbone of proteins to produce peptides of varying lengths⁶⁷. These proteins are often subjected to a separation technique prior to digestion and analysis as shown in figure 1.10. The most common technique used for the separation of proteins from biological samples includes the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which separates proteins by their molecular weight. Once separated the protein of interest is excised and digestion is performed.

Figure 1.10 Bottom-up proteomics workflow using a tryptic digestion protocol.
Created with [BioRender.com](https://www.biorender.com).

Trypsin is the protease used in the bottom-up experiments conducted in this work for several desirable reasons. Trypsin is typically used in bottom-up experiments as it is highly specific to arginine and lysine residues unless either are followed by a proline residue⁶⁹. Not only does trypsin have predictable lysis behaviour, but it also typically cleaves peptides in the mass range of 700-1500 Da which is suitable for low mass range MS analysis. Finally, the arginine and lysine residues found at the C-terminus of tryptic peptides have high proton affinities in reversed-phase solvents as shown in table 1.1. As such, peptides are more likely to hold multiple ion charges which is desirable for ion detection and sequencing.

Table 1.1 Proton affinities of relevant amino acids and solvents at 298K^{70,71}.

Molecule	Proton affinity (kJ/mol)
Arginine	1051.0
Lysine	996.0
Acetonitrile	779.2
Methanol	754.2
Water	691.0
Dimethylsulfoxide	885.0

Peptides are excellent ions to observe via collision induced dissociation (CID) due to their well-known predictable ion fragmentation patterns. MS/MS can be performed on peptide precursor ions which typically dissociate via the weakest bonds, allowing for predictable peptide cleavage patterns and localization of charge. With this, a series of y-ions and b-ions can be observed under MS/MS analysis and compared to an ion database to determine the most likely protein identity as shown in figure 1.11. Peptide and Protein MS utility programs such as Protein Prospector (UCSF Mass Spectrometry Facility, 2020) can predict the digestion patterns and furthermore the product ions of said peptides for fast and easy identification. Search engines such as MASCOT (Matrix Science, 2019) search primary sequence databases using uninterpreted MS/MS data to match with predicted mass values of proteins for protein identification.

Figure 1.11 The most common CID fragmentation pattern of a peptide. Adapted from Paiz⁶⁷.

1.10 Rationale and Hypothesis

VSe1-28 was progressed as a suitable VSe candidate in combination with oncolytic virotherapy due to its enhanced stability, toxicity profiles and viral activity. For the drug to be considered a candidate for phase I human trials, *in vitro* and *in vivo* assays were needed to determine the safety, metabolic activity, and molecular target. Our collaborative group had previously found that VSe1-28 reduces the level of NFkB nuclear translocation and subsequent reduction of the formation of IFN β and other proinflammatory cytokines involved with the antiviral signaling pathway. With this evidence, the next studies included the determination and temporal dynamics of metabolites, molecular target(s), and mechanism of action of VSe1-28. To accomplish this, two main goals were established, a metabolomics study which focused on the identification of metabolites in Vero cells and a proteomics study to target and identify the molecular target and mechanism of action of VSe1-28.

Our first goal pursued via a metabolomics approach investigated the pharmacokinetic properties of VSe1-28 by monitoring its metabolomic activity via time course *in vitro* assays. It has previously been determined that GSH is not the molecular target of VSe1-28 however has been identified as an interacting molecule through ligand-based affinity capture chromatography (binding to GSTP1, data not shown). As such it was predicted that VSe1-28 would form adducts with GSH and be a possible generator of metabolites over time in our assays. Secondly, it is important to identify all possible metabolite products and their molecular structures to account for all administered VSe1-28 and to assess their safety profiles. To validate the GSH hypothesis and investigate other possible metabolites, *in vitro* assays were performed by lysing cell cultures at pre-selected time points of 0.25, 4, 8 and 24 hr and collecting lysate and growth media for LC-MS analysis.

The second goal focused on identifying the molecular target and mechanism of action of VSe1-28. It is well described in literature that various drugs interact with NFkB through covalent modification, including sesquiterpene lactones and epoxyquinone A monomers which bind to Cys38 in the DNA binding region of the p65 subunit of NFkB. Another drug of high interest to our group is dimethyl fumarate (DMF) which has been demonstrated by our group to inhibit nuclear translocation in a similar manner to the suspected molecular target and mechanism of action of VSe1-28. As DMF possesses a similar α , β unsaturated carbonyl moiety to VSe1-28, they both can undergo Michael addition to biologically active cysteine residues. The p65 subunit only possesses 2 biologically active cysteines being Cys120 and Cys38 and however VSe1-28 is suspected to bind to the Cys38 like DMF. To confirm this hypothesis, a six-length amino acid

sequence of the biologically active Cys38 region was synthesized to develop an MRM method capable of identifying VSe1-28 modified Cys38 in a digested p65 *in vivo* and/or *in vitro* samples. Furthermore, MS/MS level scans were employed to confirm the positive identification of extracted p65 protein by MASCOT database.

Chapter 2: Materials and Methods

The following is a general description of the materials and methods used throughout various experiments conducted in this thesis. Experiments deviating from the materials and methods below will be elaborated in subsequent chapters.

Pierce trypsin protease, formic acid, HPLC and LCMS grade water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lyophilized hemoglobin porcine, caffeine, 1,4-dithiothreitol (DTT), iodoacetamide were purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents, compounds, glassware, and instrumentation used in section 2.3 were prepared and provided by University of Ottawa and sections 2.1, 2.4, and 2.5 were prepared and provided by Ottawa Hospital Research Institute (OHRI).

Special instruments used include a Dionex 3000 Ultimate HPLC system (Thermo Scientific, Waltham, MA, USA), an AB Sciex 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA), an AB Sciex QStar XL mass spectrometer (AB Sciex, Framingham, MA, USA). Glassware included: Thermo Scientific NanoES spray capillary tips (Waltham, MA, USA), 2 mL amber screw vials and 250 μ L autosampler inserts (Thermo Scientific, Waltham, MA, USA), and Amicon Ultra 0.5 mL centrifugal filters with a 3 kDa nominal molecular weight limit (MilliporeSigma, Burlington, MA USA).

2.1 *In Vitro* Time Course Experiment for VSe1-28 Metabolite Identification

It is paramount to investigate the metabolite formation of a potential drug candidate to account for the full dosage throughout the pharmacokinetics process of ADME. To assess its safety and toxicity levels, an *in vitro* time course drug candidate spiking assay was developed to observe metabolite formation over time by VSe1-28. In this work, *in vitro* is defined as biological experiments conducted in cell cultures unless otherwise specified. Vero cells were plated in eight 8 x 35mm dish in 2 mL of growth media (OptiPro) for cells to reach confluency the following day. Four plates were then treated with 42 μ M of VSe1-28 from a 50 mM stock and the four remaining plates were treated with the same volume of DMSO (1.68 μ L). A concentration of 42 μ M for the VSe1-28 spike was determined based on previous toxicity studies that concluded that concentrations above 42 μ M were cytotoxic and did not provide an effective dose range for viral sensitization³¹. Growth media and lysate were collected from one VSe1-28 and one VSe1-28 dish after 0.25hr, 4hr, 8hr, and 24hr and an aliquot of each sample was placed into two tubes each.

To collect lysate, cells were washed in 0.5 mL of hypotonic buffer (20mM Tris-HCl, pH 7.4) before scraping cells using cell scraper and transferred into a 1.5 mL tube. The tubes were snap frozen for 20 minutes, thawed at 37 °C and vortexed. This process of freeze/thaw and vortex was then repeated before clarifying via centrifugation at max speed for 10 minutes. The supernatant was transferred to a new tube and used as the lysate.

2.2 Tryptic digestion of proteins

To develop an MRM method to observe the molecular target of interest with bound VSe1-28, synthesized peptides described later in section 2.3 were subjected to a standard tryptic digestion. Later, extracted p65 protein would also be subjected to a tryptic digestion described in chapter 5. Proteins which were received solubilized were dissolved in 50 mM ammonium bicarbonate and were denatured by heating at 70°C for 5 minutes on a heated shaker at 200rpm. The proteins' disulfide bridges were reduced using dithiothreitol (DTT) at a concentration of 5 mM and were heated at 56°C for 1 hour. The reduced proteins were allowed to cool to room temperature. Iodoacetamide was added until the solution consisted of 10mM concentration and incubated in the dark for 1 hour. Sequencing-grade trypsin (Promega) was re-constituted into a 50 mM ammonium bicarbonate solution and was added to each protein in a 1:20 (w/w; protease: protein) ratio. The proteins were incubated over night at 37°C.

2.3 Synthesis and covalent modification of YKCEGR

The following protocol was performed by collaborators at the university of Ottawa for our lab to develop an MRM method to monitor the formation of the suspected molecular target of VSe1-28. The synthesis of YKCEGR was performed using solid phase peptide synthesis using a CEM liberty blue synthesizer. 2-Chlorotrityl chloride resin was used for a carboxylic acid C-terminus. Standard coupling was performed using Fmoc-amino acids, coupling with Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU) and N,N-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) and N-Methyl-2-Pyrrolidone (NMP) solvent. After synthesis, cleavage off the resin was done with 88%

Trifluoroacetic acid (TFA), 5% water, 5% phenol and 2% triisopropylsilane. Cleaved peptide was then precipitated in cold ether and spun down, the supernatant decanted, and precipitated peptide was dissolved in water to be lyophilized. The cleaved peptide was then incubated with either iodoacetamide or MD3011 (1-part peptide: 1.5-part compound) in water at 37 °C for 2 hours. Extraction was performed with ethyl acetate to remove residual compound and then the modified peptide was lyophilized. This yielded a white fluffy powder.

2.4 *In vitro* VSe's treatment of p65-FLAG

2.4.1 Preparation of buffers and solutions

The following protocol was designed and conducted by collaborators at the Ottawa Hospital Research Institute (OHRI). In this subsection, *in vitro* is defined as the use of pure immuno-purified p65 protein isolated and extracted from cell cultures. The following buffers and solutions were prepared for the *in vitro* VSe's treatment of p65-FLAG samples. A 100mM stock solution of VSe1-28 (MD03011), VSe17F (DMF) and DMSO were prepared using HPLC grade H₂O. Solutions such as 100 mL of 100 mM ammonium bicarbonate (ABC) NH₄HCO₃, 0.5 M of dithiothreitol (DTT), 12% phosphoric acid and 0.2% F.A. were prepared for various sections of the experiment. 10 mL of S-TRAP solubilization buffer was prepared using 100 mM Tetraethylammonium bromide (TEAB), 10% Sodium dodecyl sulfate (SDS) to a pH of 7.1. A 10 mL solution of S-Trap buffer was prepared using 100 mM TEAB in methanol where 1 mL was diluted in 9 mL of methanol. A 10 mL digestion buffer was prepared using 50 mM TEAB, pH 8.0 where 500 µL was added to a final concentration of 1M TEAB solution in H₂O (HPLC grade). A 10mL

solution of elution buffer was prepared using 100 μ L of 20% F.A., 5 mL ACN, and 4.9 mL of H₂O (HPLC grade).

2.4.2 Covalent modification (alkylation) of p65-FLAG

Covalent alkylation modification of p65-Flag was prepared in a 1:10 molar ratio (protein: sensitizer). Once diluted in 100 μ L of 50 mM ABC the sample was divided into 3 tubes, 10 μ g of p65-FLAG was used per tube for each modification (VSe1-28, DMF and DMSO). A 10mM solution of MD03011 and DMF respectively were prepared from the 100 mM stocks outlined in section 2.4.1. and a DMSO stock was made by diluting 5 μ L of compound stock in 45 μ L DMSO. 1.6 μ L of DMF or MD03011 was then added to a tube respectively to a concentration of 160 μ M. 2 μ L of DMSO was added to the final tube. Once added, tubes were incubated for 90 minutes at room temperature. Following this, 33 μ L of solubilization buffer was added to each tube and mixed accordingly to a final volume of 66 μ L. This sample preparation was then used in the S-TRAP digestion columns.

2.4.3 S-Trap digestion

Reagents and buffers prepared in section 2.4.1. were used for p65-Flag modification, binding and digestion. Trypsin stock was prepared fresh in a 1 μ g/ μ L concentration in HPLC-grade H₂O. S-Trap™ micro columns ($\leq$ 100 μ g) Universal MS sample prep were used.

Following section 2.4.2., the 66 μ L of solubilized protein was ready for binding and was loaded onto the S-TRAP column along with 7 μ L of 12% phosphoric acid and 230 μ L of S-TRAP buffer, mixed well then spun at 4000 rpm for 1 minute. The column was

then washed with 500 μL of S-TRAP buffer and spun again at 4000 rpm for 1 minute before changing the collection tube. This process was repeated 3x to bind all protein. Column washed with 500 μL of S-TRAP buffer and spun at 4000 rpm for 1 minute before changing the collection tube.

The denatured, trapped and cleaned protein was then subjected to digestion and elution from the column. A 25 μL aliquot of trypsin solution (dilute 2 μL of trypsin stock solution in 98 μL of digestion buffer) was added to the S-TRAP columns and was incubated for 1.5 hours at 47 $^{\circ}\text{C}$. 80 μL of digestion buffer was then added, spun down at 4000 rpm for 1 minute and the first fraction was discarded. The collection tube was changed, labelled fraction 2 and 80 μL of 0.2% F.A. was added, spun down at 4000 rpm for 1 minute. The collection tube was changed, labelled fraction 3 and 80 μL of elution buffer was added, spun down at 4000 rpm for 1 minute. Fraction 3 was evaporated via speedvac and pulled together with fraction 2.

Figure 2.1. S-TRAP sample processing procedure for p65 and p65 modified proteins. Reproduced from Profti⁷².

2.5 *In vivo* VSe treatment of p65-FLAG-transfected 293T cells

2.5.1 Transfection of p65-Flag and *in vivo* treatment of p65-Flag expressing 293T cells

The following protocol was designed and conducted by collaborators at the Ottawa Hospital Research Institute (OHRI). In this subsection, *in vivo* work is described as experiments conducted on cell cultures. Over a period of two days, transfection of p65-Flag was conducted using 7 µg of plasmid per 10 cm dish. The plasmid used was Human RELA/Transcription factor p65/NFkB, p65 ORF mammalian expression plasmid, C-Flag tag (pCMV3-ORF-FLAG) from Sino Biological. The media used was a mixture of Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and 1% antibiotics.

Nine 10 cm dishes were seeded with 293T cells for *in vivo* VSe treatment with 6×10^6 cells/dish. The following day 63 µL of pCMV3-p65-FLAG was diluted in 13.5 mL of Opti-MEM and 180 µL of Lipofectamine 2000 in 13.5 mL of Opti-MEM and incubated for 5 minutes at room temperature. The DNA solution was slowly added to Lipofectamine 2000 solution and incubated again for another 20 minutes. The media in the nine dishes were then changed to 10 mL of full DMEM and 3 mL of the DNA-Lipofectamine solution were added to each dish and incubated for 1 day. On the third day the media was changed.

On the fourth day the *in vivo* treatment of p65-Flag expressing 293T cells was conducted using the following reagents, DMF (VSe17F) 100 mM stock solution, MD03011 (VSe1-28) 100 mM stock solution and DMSO. Lysis (binding) buffer was prepared using 50 mM Tris HCl at pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and HPLC grade water.

The VSe's solutions were then prepared for treatment, by preparing 30 mL of full DMEM containing 45 μ L of 100 mM DMF (VSe17F) to a final concentration of 150 μ M. 30 mL of full DMEM containing 15 μ L of 100 mM MD03011 (VSe1-28) to a final concentration of 50 μ M. Prepare 30 mL full DMEM containing 45 μ L DMSO. The culture media was then removed, and the cells were washed with phosphate-buffered saline (PBS). For VSe treatment, 10 mL of DMEM-VSe solution was added per plate in biological triplicates and incubated for 2 hours in the incubator. The cells were then lysed by adding 1 mL of lysis buffer supplemented with protease inhibitor cocktail. The extracts were then pulled together from the biological triplicates.

2.5.2 P65-FLAG Purification

The following buffers and solutions were prepared prior to P65-FLAG purification. A washing buffer using tert-butyldimethylsilyl (TBS) at pH 7.6, 1L /500 mL, 6.05 g/ 3 g Tris, 8.76 g/ 4.37 g NaCl, elution buffer using 0.1 M glycine HCl at pH 3.5, 200 mL/100 mL, 1.5 g/ 0.75 g, neutralization buffer using 1 M Tris at pH 8.0, 200 mL/100 mL, 24 g/ 12 g, and a storage buffer using 0.02% sodium azide. Anti-Flag M2 Affinity Gel (A2220, Sigma) was also required for the p65-FLAG purification process.

For column preparation, the column was prepared by placing 500 μ L of anti-Flag M2 Affinity Gel to three clean new columns and adding 1 mL of washing buffer to wash away storage solution by gravity flow. Gel was washed with 1.5 mL of elution buffer followed by 3-5 mL of washing buffer. The column was then pre-equilibrated with 3 mL of lysis buffer. To bind the FLAG fusion protein the column 2 mL of cell lysate was loaded onto each respective column under gravity flow. The flowthrough was collected and

reloaded onto the column multiple times to improve the binding efficiency. The remaining flowthrough was kept for washing buffer. The column was then washed with 12 mL of washing buffer to remove any proteins that are not bound to the M2 antibody and allowed to drain completely.

Following binding and washing, bound p65-FLAG was eluted from the column with three 1.5 mL aliquots of elution buffer into three collecting tubes containing 25 μ L of neutralization buffer. 25 μ L of each elution fraction was kept for washing buffer with M5 and/or p65 antibody. The two highest concentrated fractions were pooled together.

The column was then regenerated immediately after use by washing the column with 3 mL of elution buffer, re-equilibrated with 6 mL of washing buffer until the effluent was at a neutral pH. To store the column, it was then washed with 2-5 mL of column storage buffer and then another 2 mL was added and stored at 2-8°C. On the 6th-7th day washing buffer was added for all FLAG-tag and/or p65 fractions and the best elution fractions were selected for analysis.

2.5.3 Buffer exchange, concentrating and sample preparation

Similar buffers used in section 2.4.1. were used for buffer exchange, concentrating and sample preparation. The buffers and solutions used were 100 mM ABC, S-TRAP solubilization buffer. This was prepared using a buffer exchange of 30 μ g of p65-FLAG against 50mM ABC using a slide-A-lyser cassette (MW cut-off 3000) at 4°C overnight with 3 buffer changes. P65-FLAG samples were then concentrated using Amicon Ultra-0.5 centrifugation filters for 4 minutes at 14,000 rpm. The protein was concentrated to a final volume of 250 μ L. The protein concentration was subjected to a Qubit protein assay

(Sigma Aldrich). 10 µg of p65-FLAG protein was diluted in 100 µL of 50 mM ABC to three 1.5 mL tubes. 100 µL of solubilization buffer was added to each tube and mixed to yield a final volume of 200 µL. This sample preparation was then used in the S-TRAP digestion columns.

2.5.4 S-Trap digestion

The binding and digestion protocol follow similar steps as described above in the *in vitro* p65-Flag modification protocol. To bind to the column, 350 µL of protein added to S-TRAP column along with 20 µL of 12% phosphoric acid and 700 µL of S-TRAP buffer and spun at 4000 rpm for 1 minute. This process was repeated 3x to bind all protein. Column washed with 500 µL of S-TRAP buffer and spun at 4000 rpm for 1 minute before changing the collection tube.

2.6 Spin filtering

To analyze VSe time course experiments separation of the small molecules from large biomolecules was required before LC-MS analysis to avoid high pressure issues. To perform this, growth media and lysate spiked VSe1-28 samples were passed through the spin filters (Millipore Corp.) with a molecular cutoff of 3kDa (Figure 2.2). The Amicon Ultra-0.5 filter was added to a microcentrifuge tube. 350 µL of sample was added to the filter and capped. The capped collection tube was centrifuged at 14,000 g/min for 30 minutes at 4°C. VSe samples were stored frozen at -80°C until LC-MS analysis⁷³.

Figure 2.2 Spin filtering to isolate VSes from complex biological samples. Steps (1) and (2) were applied to achieve VSe metabolites. Steps (3) and (4) were not needed for the purpose of this experiment. Adapted from Millipore⁷⁴.

2.7 Direct Infusion MS

MS analysis of VSe1-28, internal standard caffeine, modified GSH and digested p65 proteins were analyzed via direct infusion on the hybrid triple quadrupole time-of-flight (QqTOF) QStar XL mass spectrometer (AB Sciex). Analysis was performed on a NanoESI spray capillary (Thermo Scientific) with ~6-7 μ L loaded per experiment dissolved in a solution of HPLC grade H₂O and 0.1% F.A. The tip was gently bent and cracked against the charged curtain plate to enable analyte solution spray into the MS. The most common settings used for direct infusion experiments used a declustering potential of 30 V, focusing potential of 120 V, a CID gas setting of 2, and an ion gas source setting of 1. Ion spray voltage varied depending on the solution of interest, where ion spray voltage varied between 1000-1500 V. Full mass scans were performed on all samples to develop MRM methods and product ions of interest by optimizing collisions energy. These MRMs were applied later in section 2.8.1.

2.8 LC-MS/MS

Most of the metabolomics and proteomics experiments of VSe1-28 were performed on the AB Sciex 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer or the Agilent 6546 QTOF mass spectrometer using RP-HPLC to separate the biological samples and solutions. Both instruments were equipped with an ESI source coupled to the HPLC system and used positive ion mode for analysis. The solvents selected for RP-LC analysis were 0.1% F.A. as solvent A and 0.1% F.A. in ACN as solvent B.

2.8.1 LC-MS/MS for Metabolomics

Samples for metabolomics analysis were analyzed via LC-MS in the following manner. A biological sample was prepared following section 2.6 where an internal standard was added prior to filtration and transferred into an amber vial with a conical spring insert and placed in the HPLC autosampler tray for analysis. 20 μ L of sample was then injected and was separated using a Phenomenex C18 150 x 4.60 mm column with a 5 μ m particle size and with a metabolomics designed gradient shown in figure 2.3.

On the QTRAP 4000, the analytes were acquired between a mass range of 50 – 1500 Da. In the MRM method, VSe1-28 and the internal standard caffeine were monitored by quantitative and qualitative transitions. The method for metabolite analysis named “3011 Caf 18 min w MRM Q3” performed MS level analysis followed immediately by MRM analysis. MRM Q1 $\rightarrow$ Q3 transitions selected for monitoring are listed in table 2.1. The enhanced mass scan function provided by the LIT was not operational therefore MS level scans were employed.

Figure 2.3 Metabolomics gradient used for the analysis of biological samples and VSe1-28 on the QTRAP 4000 and Agilent 6546 QTOF MS.

Metabolomics analysis conducted on the Agilent QTOF 6546 was conducted on a Polaris C18-A 100 x 2.0 mm with a 3 μ m particle size and used the same gradient shown in figure 2.3. The QTOF was set to acquire masses between the range of 50 – 1500 Da. The MS level method used for quantitative analysis was a named “Digested Peptides” and the qualitative method “Digested Peptides Auto MS/MS” used Auto MS/MS, triggered by an ion abundance parameter of 20, 000 counts/spectrum. Both instruments used an electrospray voltage of 3500 V. For the “Digested Peptides Auto MS/MS” method collision energy was varied based on a collision formula ranging from 10 eV to 40 eV as shown in figure 2.4.

Figure 2.4 Collision energy formulas for digested peptide Auto MS/MS method for the analysis of the time course experiments.

2.8.2 LC-MS/MS for Proteomics

Sample preparation was performed similarly to section 2.8.1. A proteomics LC-MS method was developed by multiple optimization experiments using a standard digest of porcine hemoglobin (Sigma Aldrich) for peptide identification. The LC gradient was developed based on Agilent Technologies notes⁷⁵ and is shown in figure 2.5. It was determined that the optimal collision energies for Auto MS/MS should be performed by a collision energy formula feature on MassHunter as shown in figure 2.6. The mass scan range was set to 100-1600 Da with an Auto MS/MS mass exclusion of 100 – 250 Da to ensure MS/MS analysis focused on peptide masses of interest.

MASCOT (Matrixscience, Boston, MA, USA) database searches were conducted using the Ion MS/MS Search query for protein identification. The SwissProt protein database was selected for all searches using the following parameters, fixed variable modification of cysteine residues via carbamidomethylation, variable modifications of the oxidation of methionine, peptide mass tolerance of 1.2 Da and an MS/MS tolerance of 0.2 Da. Peptide charge search was selected for $[M+2]^{+2}$, $[M+3]^{+3}$, and $[M+4]^{+4}$ charged peptides with up to two missed cleavages allowed for tryptic digestion.

Figure 2.6 Proteomics gradient used for the analysis of VSe1-28 and peptide analogues on the Agilent 6546 QTOF MS.

Figure 2.5 Collision energy formulas used to determine the optimal collision energies for differently charged peptides.

Chapter 3: MRM Development

MRM provides a highly specific and sensitive technique for quantification of analytes in complex matrices⁷⁶. This technique is often referred to as tandem MS (MS/MS) and is used in clinical laboratories to provide a specific identification and quantification method for drugs of interest⁷⁷. Although MRMs provide superior results over full mass scans, method development and optimization are time-consuming processes. Some instruments, such as the QTRAP 4000 can perform direct infusion compound optimization parameters however manual compound optimization is often still preferred. Typically in MRM studies, there is a quantitative transition used for quantification of analyte and a qualitative transition used as a secondary positive identification of the analyte of interest. The addition of a qualitative transition increases the likelihood of the quantitative transition belonging to the ion of interest so long as the chromatographic elution profiles of both transitions are identical.

The first MRM development study was conducted to quantify the decrease of VSe1-28 more accurately in cells due to the enhanced capabilities of the TQMS instrument. To do this, VSe1-28 was dissolved in HPLC grade water with 0.1% F.A and electrosprayed onto the QTOF (QSTAR, Sciex) instrument for full scan and product ion analysis. The second VSe1-28 study focused on the qualitative analysis compound identification of VSe1-28 bound to the suspected binding location in the p65 subunit. To do this, VSe1-28 and iodoacetamide was incubated with a short six amino acid sequence peptide YKCEGR, to generate 3 samples, only peptide (YKCEGR), iodoacetamide bound (YKC(Alk)EGR), and VSe1-28 bound (YKC(VSe1-28)EGR). The first MRM study was validated via LC-

MS on the QTRAP 4000 while the second MRM study was not validated due to instrument failure and complications.

The internal standard selected for quantitative LC-MS analysis of VSe1-28 was caffeine. Caffeine was selected as the internal standard based on previous qualitative control (QC) studies which determined it had high reproducibility, as well as a desirable retention time (RT), did not overlap with VSe1-28. Caffeine also demonstrated long-term stability, was readily available, inexpensive and used extensively in the literature, making it a desirable candidate as an internal standard³². The internal standard concentration of 0.25 μ M was continued following previous suggestions for QTRAP 4000 experimental analysis³². The precursor-product ion transitions pairs selected for MRM of caffeine were m/z 195.0882 $\rightarrow$ m/z 138.200 and m/z 195.088 $\rightarrow$ m/z 110.100 for quantitative and qualitative monitoring, respectively. The quantitative and qualitative transition ions selected for caffeine are listed in table 2.1 in subsection 2.8.1. of materials and methods. As caffeine has previously been subjected to numerous qualitative control and MRM studies³², caffeine was readily and easily accepted as the continued internal standard for time course experiments described in chapter 4. Not only was caffeine used as an internal standard for quantitation, but it was also used as an indicator of the instrument's performance which was later used to demonstrate a dramatic reduction in performance post fire damage in our lab.

3.1 LC-MRM for the monitoring of VSe1-28 metabolism

The characteristic chlorine isotope pattern of a singly chlorinated compound is a 3:1 ratio while that of a doubly chlorinated 9:6:1. VSe1-28 contains two chlorine atoms when in solution as shown in figure 3.1 and therefore, we should expect that VSe1-28 has a doubly chlorinated isotope pattern. As it is known, within mass spectra, chlorinated compounds have a natural isotope ratio of 33% or 66% $[M+2]^+$ as present in figure 3.2. As expected, in figure 3.2, the precursor ion mass of m/z 281.0478 which was comparable to its expected mass of m/z 281.0459 is found along with its characteristic m/z 283.0454 single and m/z 285.0438 double ^{37}Cl isotope distribution. To develop an MRM, a full mass scan of VSe1-28 was performed on the QSTAR XL. This full scan confirmed the presence and accurate mass identity of the precursor ion. Following precursor identification, it was subjected to a product ion scan and the collision energy was increased stepwise to determine and optimize the two highest intensity peaks for transition ions.

Figure 3.1 Structures of VSe1-28 (a) and internal standard caffeine (b).

The two highest intensity fragment ions produced by the product ion scan of VSe1-28 m/z 281.0459 were m/z 177.9808 and m/z 100.0808, respectively (figure 3.3). The possible structures of the two fragments were investigated and it was determined that the masses were from the internal fragmentation of VSe1-28 as shown in figure 3.4. The larger internal mass fragment, m/z 178.9540 was determined to be the chlorinated warhead of VSe1-28 while m/z 100.0808 was the morpholine structure. With this determination, the product ion fragments were compared with their expected masses of m/z 178.9540 and m/z 100.0762 and were determined to be comparable. Although the morpholine-like structure of m/z 100.0808 was a good candidate as a qualitative transition ion a more desirable candidate was selected for its chlorine isotope pattern, m/z 283.0400. As there is a low abundance of chlorinated compounds *in vivo*, the presence of VSe1-28 and/or its metabolites may be elucidated by scanning mass spectra for ions that bear a chlorinated M+2 isotope pattern.

The optimal collision energy for each fragment was determined by performing a product ion scan on the precursor ion. The multiple count accumulation (MCA) mode which sums the acquired spectra over time was turned off to optimize the collision energy which produced the highest intensity fragments. The optimization was performed in a stepwise fashion where collision energy was increased in increments of 5 eV, once a 10 eV range was determined the stepwise process was repeated with 2 eV increments. A thorough investigation of the precursor and product ions of VSe1-28 table 3.1 was developed for future MRM transitions applied in chapter 4 of this work.

Figure 3.2 Direct infusion full mass scan of VSe1-28.

Figure 3.3 Product ion scan of VSe1-28 parent (m/z 281.0459).

Figure 3.2 VSe1-28 mechanism of internal fragmentation. Fragment (a) being m/z of 100.0762 and fragment (b) being m/z of 178.9540.

Table 3.1 MRM parameters for the quantitative and qualitative transition ions of VSe1-28.

Compound	Quantitative Transition		Collision Energy (eV)
	Parent Ion (m/z)	Fragment Ion (m/z)	
VSe1-28	281.0459	177.9803	25
Compound	Qualitative Transition		Collision Energy (eV)
	Parent Ion (m/z)	Fragment Ion (m/z)	
VSe1-28	283.0400	179.9803	25

3.2 LC-MRM for VSe1-28 MOA confirmation

It has been determined through previous studies that DMF binds to Cys38 within the DNA binding region of the p65 subunit of NF- κ B transcription factor²⁰. Our hypothesis follows the same assumption that VSe1-28 will bind to Cys38 and inhibit nuclear translocation and subsequent antiviral response. It has been hypothesized that, while peptides are cleaved with trypsin at a lysine or arginine residue, the covalent modification of VSe1-28 inhibits the cleavage of lysine resulting in a missed cleavage. As such, the resulting peptide chain expected for the binding of VSe1-28 to Cys38 is YKCEGR. Following this, to confirm our hypothesized mechanism of action, YKCEGR was synthesized following the protocol stated in chapter 2, subsection 2.3, synthesis and covalent modification of YKCEGR.

To observe the mechanism of action, the short peptide sequence YKCEGR required an MRM method with 3 possible variations. The first variation being unbound YKCEGR, secondly VSe1-28 bound (YKC(VSe1-28)EGR), or thirdly iodoacetamide bound (YKC(Alk)EGR) as shown in figure 3.5. Iodoacetamide was selected as a variation due to its use in the standard tryptic digestion protocol, possibly alkylating unbound YKCEGR during digestion. Upon synthesis and incubation of YKCEGR with VSe1-28 or iodoacetamide for cysteine modification, the QSTAR XL was used to validate the successful synthesis of the 3 peptides through MS + MS/MS analysis.

Figure 3.3 Structure of YKCEGR (a), YKC(Carbamidomethyl)EGR (b), and YKC(VSe1-28)EGR (c) modifications for LC-MS/MS analysis.

A critical step in designing an MRM method involving peptides begins with identifying the target peptide (precursor ion) and the corresponding product ions based on a general set of criteria. These criteria are: selecting two to four transitions for a target, selecting singly charged y product ions, should be a predominant product ion and avoiding product ions with similar m/z to precursors ions (higher m/z than precursor is preferred)⁷⁶. When possible, these criteria were followed.

To analyze the peptides on the QSTAR XL, the lyophilized white powders of YKCEGR, YKC(VSe1-28)EGR and YCK(Alk)EGR were dissolved in separate scintillation vials in HPLC grade water with 0.1% F.A. for analysis. Prior to analysis a list of peptide m/z were collected as shown in table 3.2 using ProteinProspector MS-Product Ion feature which was also used to predict potential y-ions and b-ions upon fragmentation analysis.

Table 3.2 List of YKCEGR and modified YKCEGR peptide m/z for analysis.

<i>Peptide</i>	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
YKCEGR	755.3505	378.1789	252.4550
YKC(VSe1-28)EGR	999.4124	500.2101	333.8093
YKC(Alk)EGR	812.3719	406.6896	271.4622
CEGR	464.1922	232.5997	---
C(VSe1-28)EGR	708.2536	354.6307	---
C(Alk)EGR	521.2137	261.1105	---

6.0 μ L of sample was loaded onto a nanoESI spray tip and sprayed on the QSTAR XL to collect a full MS scan of each peptide. The full scan of YKCEGR revealed three charged states, $[M+H]^+ = m/z$ 755.3354, $[M+2H]^{2+} = m/z$ 378.1650 and, $[M+3H]^{3+} = m/z$ 252.4434. (figure 3.6) which closely matched the expected masses of $[M+H]^+ = m/z$

755.3505, $[M+2H]^{2+} = m/z$ 378.1789 and, $[M+3H]^{3+} = m/z$ 252.4550. To further verify that this was the correct peptide, the precursors were subjected to product ion scans and their spectra were compared against the y-ions and b-ions predicted by ProteinProspector, confirming the correct synthesis of the peptide. To develop MRMs the following method was conducted for all three peptides; the most predominant precursor was selected and subjected to a product ion scan using various collision energies to confirm the highest yield and intensity fragment ions for transitions. The quantitative transition selected for the MRM scans of YKCEGR were m/z 378.1789 $\rightarrow$ m/z 464.1979 with a collision energy of 21 eV and the qualitative transition being m/z 252.4550 $\rightarrow$ m/z 296.6492 with a collision energy of 14 eV (data not shown).

Figure 3.4 MS of YKCEGR (m/z 755.3505) and its singly, doubly and triply charged states.

In the same manner, YKC(VSe1-28)EGR and YKC(Alk)EGR were loaded onto a nanoESI spray tip and sprayed on the QSTAR XL to collect a full MS scan. The full scans of YKC(VSe1-28)EGR and YKC(Alk)EGR revealed the expected three charged states that match the expected m/z found in table 3.2. The quantitative transition ion pair selected for the MRM scans of YKC(VSe1-28)EGR were m/z 552.2009 $\rightarrow$ m/z 473.2103 Da with a collision energy of 26 eV and the qualitative transition ion pair being m/z 333.8093 $\rightarrow$ m/z 409.6806 Da with a collision energy of 19 eV as shown in figure 3.7 and 3.8. For YKC(Alk)EGR, the quantitative transition ion pair selected for the MRM scans were m/z 406.6896 $\rightarrow$ m/z 521.2137 with a collision energy of 24 eV and the qualitative transition ion pair being m/z 271.4519 $\rightarrow$ m/z 325.1624 with a collision energy of 15 eV. The m/z of the ions found in the 3.7-3.10 spectra closely matched the predicted m/z table 3.2 and. In summary, table 3.3 and 3.4 were developed following the meticulous collection of full scans and product ions to find the best transition ion pairs for MRM.

Figure 3.5 Full mass scan of YKC(VSe1-28)EGR (m/z 999.4124) and its doubly and triply charged states.

Figure 3.6 Product ion of the doubly charged precursor ion YKC(VSe1-28)EGR (m/z 500.2009).

Figure 3.7 Full mass scan from 300 - 600 Da of YKC(Alk)EGR in its doubly charged state (m/z 406.6896) charged state.

Figure 3.8 Product ion of the doubly charged precursor ion YKC(Alk)EGR (m/z 406.6896).

Table 3.3 Quantitative transitions used for YKCEGR and modified peptide MRM parameters to monitor the MOA of VSe1-28 in biological samples.

Compound	Quantitative Transition		Collision Energy (eV)
	Parent Ion (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)	
YKCEGR	378.1789	464.1979	21
YKC(VSe1-28)EGR	552.2009	473.2103	26
YKC(Alk)EGR	406.6896	521.2137	24

Table 3.4 Confirmatory transitions used for YKCEGR and modified peptide MRM parameters to monitor the MOA of VSe1-28 in biological samples.

Compound	Confirmatory Transition		Collision Energy (eV)
	Parent Ion (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)	
YKCEGR	252.455	296.6492	14
YKC(VSe1-28)EGR	333.8093	409.6806	19
YKC(Alk)EGR	271.4519	325.1624	15

3.3 Iodoacetamide Discovery

Following the development of the MRM method for YKCEGR and modified variations, the three peptides were subjected to a standard tryptic digestion to develop another MRM method specific to post-digestion modification identification. This study both investigated if YKCEGR cleaves at the lysine residue with VSe1-28 bound and the possible product ions for transitions from CEGR. Following the standard tryptic digestion protocol described in chapter 2 subsection 2.2, 20 μ L of each peptide was injected onto the QTOF 6546 using the digested peptides LC-MS method. The EIC's for all peptides were performed on the three positively charged precursor states where a positive identification of CEGR and C(Alk)EGR were observed.

Interestingly, this was not the case for C(VSe1-28)EGR upon performing an extracted ion search. As shown in figure 3.11, C(VSe1-28)EGR was not present in the three possible charged precursor states and further investigation of the MS and MS/MS level scans yielded the same result. Further investigations also determined the disappearance of the full-length modified peptide YKC(VSe1-28)EGR. When a search was performed on YKC(Alk)EGR masses, it was positively identified through EICs (figure 3.12) and MS + MS/MS scans. With the identification of YKC(Alk)EGR it was discovered that iodoacetamide performed an addition-elimination reaction during the tryptic digestion.

Figure 3.9 EICs of the three possible charged states of C(VSe1-28)EGR post tryptic digestion.

Figure 3.10 EICs of the three charged states of the precursor ion YKC(Alk)EGR found in the YKC(VSe1-28)EGR tryptic digestion.

In the proposed addition-elimination mechanism shown in figure 3.10, the thioether bond present between the VSe1-28 and cysteine is weakly nucleophilic and capable of attacking the α -carbon of iodoacetamide. Once the thioether is bound to the iodoacetamide structure, VSe1-28 becomes a good electrophile and is attacked by water, eventually forming an -OH bond. In the process of -OH addition, two electrons are pushed up onto the oxygen creating a negative charge. With the thioether now positively charged, VSe1-28 becomes a good leaving group and is eliminated. This newly eliminated warhead was found in the spectrum with a m/z of 263.0798 and with the expected single chlorine isotope pattern as shown in figure 3.14.

Upon reviewing literature, it was found that overalkylation of tryptic digestions can cause the removal of PTM's of cysteine residues as well as alkylate many other sites. In another study, iodoacetamide was added to a digest as an inhibitor for unreactive cysteine sites from further glutathionylation and remained in solution for the duration of the experiment. Upon ESI-MS the digest revealed several peaks with an increase mass of 57 Da, the mass of iodoacetamide reacting with thiols and other amino groups⁷⁸. Another study demonstrated that iodoacetamide alkylation can result in a mimicked neutral loss of phosphoric acid which closely resembles the proposed mechanism in figure 3.13⁷⁹. Furthermore, a systematic evaluation of protein reduction and alkylation study was performed and revealed unspecific iodo-alkylating reactions⁸⁰, additionally supporting our findings.

Figure 3.11 Proposed mechanism for the nucleophilic addition of iodoacetamide and subsequent elimination of VSe1-28 from the cysteine residue producing the m/z of 263.0798 (a) m/z of 812.3719 (R = C₆H₁₂NO) (b).

Figure 3.12 MS level scan demonstrating the unique M+2 chlorine isotope signature of the eliminated VSe1-28 warhead as shown in figure 3.13 as structure 263.0798 (a).

Although DTT was not assumed to be the culprit in the removal of VSe1-28 from the thiol, it was removed as a precaution and a simplified tryptic digestion procedure was developed. The modified trypsin protocol went as followed, proteins were solubilized in 50mM of ABC and were denatured by heating at 70 °C for 5 minutes on a heated shaker at 200rpm. Sequence-grade trypsin (Sigma Aldrich) was re-constituted into a 50mM ABC solution and was added to each protein in a 1:20 (w/w; protease: protein) ratio. The proteins were incubated over night at 37°C.

Following the simplification of the tryptic digestion protocol, the three peptides were digested again and analyzed by LC-MS on the QTOF 6546. The precursor ion of YKC(3011)EGR was successfully determined in both the singly and doubly charged state as shown in figure 3.12. With the positive identification of the precursor ion, a product ion scan was conducted on the three peptides on the QSTAR XL and produced the MRM post-digestion CEGR method seen in table 3.5.

Figure 3.13 EICs of the two predicted digested peptides of C(3011)EGR after simplification of tryptic digestion protocol.

Table 3.5 Quantitative transition ions used for CEGR and modified peptide MRM parameters to monitor the MOA of VSe1-28 in biological samples.

Compound	Quantitative Transition		Collision Energy (eV)
	Parent Ion (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)	
CEGR	464.1922	361.1830	18
C(3011)EGR	708.2536	495.1235	22
C(Alk)EGR	521.2137	365.1127	22

The MRM peptide methods developed may only be used for qualitative analysis unless isotopically labelled internal standards (or a label free version) are synthesized, quantified and compared against the results produced from peptide analysis^{81,82}. The MRM transitions were not validated using LC-MS with the QTRAP 4000 due to an unprecedented fire at Carleton university. For future work involving the mechanism of action of VSe1-28 binding to YKCEGR, the quantitative and qualitative transition ions have been selected for experimental analysis.

3.4 Conclusions

A proposed internal fragmentation pattern for VSe1-28 is described. The two most intense product ions for the precursor ion of VSe1-28 were selected as the quantitative and qualitative transitions for MRM method development. The collision energies were optimized on the QSTAR XL and validated on the QTOF 6546.

The synthesis and incubation of YKCEGR with VSe1-28 or iodoacetamide for modification was successfully completed and validated with QTOF-MS/MS. Covalent modification of VSe1-28 and separately iodoacetamide to the cysteine of YKCEGR has been demonstrated successfully and MRM transitions have been developed for all three peptides. An important discovery was made, where iodoacetamide was replacing VSe1-28 on the cysteine. This was an important finding in developing modified digestion protocols on future VSe1-28 modified samples.

With the development of MRM transitions for pharmacokinetic, metabolomic and proteomic experiments, studies were subsequently conducted on complex biological samples, *in vivo* and *in vitro*, as described in chapters 4 and 5.

Chapter 4: Metabolomics

Pharmacokinetics (PK) is the study of the absorption, distribution, metabolism, and excretion (ADME) of a compound. In the drug discovery process, once the early drug discovery process is complete, various preclinical studies must be completed to reach phase I human trials of clinical development. As such, *in vivo* and *in vitro* assays, ADME, drug delivery and dosage are on the list of studies required for approval. Preclinical studies require the determination of safe *in vitro* and *in vivo* activity in at least one cell culture experiment and two animal species to estimate a safe dosage as well as potential metabolites which may be involved in toxicity of organs and cells. In this work, an *in vitro* time course assay was conducted to determine the metabolic activity and fate of VSe1-28 by evaluating the half-life and the identification of VSe1-28 metabolites, intracellular target, unintended target and mechanism of excretion.

The enhanced strengths of tandem mass spectrometry LC-MS/MS in the medicinal chemistry applications provides enhanced specificity, because of the capabilities of MRM methods. LC-MS/MS analysis also provides information rich detection where quantitative and qualitative results can be obtained from an experiment⁸³. As such, performing metabolomics experiments was preferred on the QTRAP 4000 for its TQMS-LIT functionalities. The first VSe1-28 time course experiment followed the materials and methods outlined in chapter 2 subsection 2.1 *in vitro* time course experiment for VSe1-28 metabolite identification and was conducted on the QTRAP 4000. Briefly, four identically prepared Vero cell culture dish samples were spiked with 42 μ M VSe1-28, the first dish was lysed and collected as a time point at 15 minutes, the second dish at 4 hrs, the third dish at 8 hrs and the fourth dish at 24 hrs. This method defined all 4 dishes as being a

singular biological set which was subjected to a technical triplicate by injecting each time point sample thrice. The second VSe1-28 time course experiment was conducted on the QTOF 6546 due to the fire in Carleton university which damaged the QTRAP 4000. Briefly, twelve identically prepared Vero cell culture dish samples were spiked with 42 μ M VSe1-28, the first three dishes were lysed and collected at a time point of 15 minutes, the second set of three dishes at 4 hours, the third set of three dishes at 8 hours and the fourth set of dishes at 24 hours. This method defined each time point as being a biological triplicate, where those three dishes were considered biologically different from each other. Each dish within the set was subjected to a technical triplicate by injecting each time point sample thrice, in total collecting nine data points for one time point.

Accounting for error, the calibration curve of VSe1-28 normalized to the internal standard caffeine averaged the technical triplicate of each the each concentration prepared by serial dilution. The standard error of the calibration curve was calculated by summing the standard error from VSe1-28 and caffeine and is represented as error bars seen in figures 4.1 and 4.6. The first VSe1-28 time course experiment averaged the technical triplicate to produce the mean and standard error was calculated for each time point and are represented as error bars seen in figures 4.2 and 4.3. The mean results of the second VSe1-28 time course experiment were conducted by averaging the technical triplicate of each dish then taking the average of the biological triplicate. The standard error was calculated by summing the standard error of each biological sample and are represented as error bars seen in figures 4.6 and 4.7.

4.1 QTRAP 4000 Time Course Experiment

Preliminary investigations for VSe1-28 and caffeine were conducted on the QTRAP 4000 to determine retention times and develop a calibration curve for time course experimental analysis. VSe1-28 was determined to have an RT of 4.9 min $\pm$ 0.3 min and caffeine an RT of 5.7 min $\pm$ 0.2 min (data not shown). For the selection of concentration range a stock solution of VSe1-28 was used to determine the linear dynamic range of the instrument by serial dilution. This led to an excellent spectral resolution and good signal to noise ratio for the analytes of interest with a wide linear dynamic range of 0.05 μ M – 42 μ M. The standard curve produced from this study showed a strong linear fit ($R^2 = 0.99$) (figure 4.1) and an LOD of 40 fmol was achieved.

Figure 4.1 A calibration curve of VSe1-28 to normalized to 0.25 μ M internal standard caffeine performed on the QTRAP 4000.

The quantitative and qualitative transitions of VSe1-28 and caffeine permitted for a precise analysis of the progressive decrease in the Vero cell cultures. The two main

experimental interests of the time course experiments were to plot VSe1-28 over time to calculate its half-life in Vero cells and secondly, to determine major metabolites produced over a 24 hour period. The interest in the VSe1-28 half-life was important in determining its stability in kidney cells and whether any biological enzymes were metabolizing our drug. To calculate half-life, a calibration curve of VSe1-28 normalized to the internal standard caffeine as shown in figure 4.1 was used to calculate the concentrations of VSe1-28 in both lysate (figure 4.2) and growth media (figure 4.3) respectively. In the lysate results, the calculations for the concentrations past the 4-hour time point were negative indicating the concentrations went below the linear dynamic range of the instrument and calibration curve. For this reason, figure 4.2 only demonstrates the first two time points of VSe1-28 in lysate. It was determined that VSe1-28 had a half-life of 3.90 hours in lysate (0.655 μ M half-life concentration) and demonstrated an exponential decay over time. Interestingly the concentration of VSe1-28 did not significantly increase in the cells over time, with a maximum observed concentration of $\sim$ 1.30 μ M. It can be hypothesized that VSe1-28 is not observed at a high concentration in cells due to some sort of metabolic process degrading the drug or VSe1-28 is degrading in the growth media.

The normalized error was determined by analyzing the technical triplicates standard deviation and propagating the relative error by squaring then adding the relative standard errors of VSe1-28 and caffeine together, taking the square root and multiplying by the normalized average concentration. The lysate concentrations were subjected to a back-calculation dilution ($C_1V_1=C_2V_2$) to account for the 0.5 mL washing of hypotonic buffer before scraping transfer to a 1.5 mL centrifugation tube. The cumulative error of VSe1-28

in did not exceed 5% in growth media and did not exceed 7% in lysate when ignoring the relative error of the 24-hour time point of 36%. The large error can be attributed to the A.U.P. extraction being below the limit of quantitation, contributing a larger source of error than if the results were in within the linear dynamic range. Overall, the errors determined in the lysate and growth media indicated reliable results.

A predictably large concentration of VSe1-28 was found in the growth media plot (figure 4.4) and determined the half-life of 4.83 hours (19.55 μM half-life concentration) in growth media. It is plausible that within the first 15 minutes of VSe1-28 treatment that the drug does not enter the cells to a significant degree in this time span. In a previous plasma stability study, it was determined that VSe1-28 had 38.9% ± 0.52 remaining in serum after 3 hours when spiked at a concentration of 80 μM (See Appendix A)³¹. In the same study, compared to other VSe analogues, VSe1-28 was detectable using LC-MRM up to 10 hours in tumor and serum³¹. When comparing to the results present in this work, VSe1-28 was observed up until ~20-hour period in growth media (figure 4.4) however only up to 5 hours in lysate. This could be because VSe1-28 was injected intratumorally in the plasma stability study while in the cell culture study VSe1-28 was not directly spiked into cells. Contrary to the results of the plasma stability study, VSe1-28 had 67% remaining in growth media after 3 hours when spiked at a concentration of 42 μM . Considering the VSe1-28 plasma stability study was conducted in mouse serum³¹ while the time course experiment was conducted in OptiPro serum free media, there could be compounds present in the mouse serum which contributed to a faster degradation.

Figure 4.2 VSe1-28 normalized to 0.25 μM internal standard caffeine monitored by the QTRAP 4000 in lysate over a period of 24 hours. Concentrations past 4 hours were below the limit of detection of the instrument and could not be plotted.

Figure 4.3 VSe1-28 normalized to 0.25 μM internal standard caffeine monitored by QTRAP 4000 in growth media over a period of 24 hours.

4.2 QTOF 6546 Time Course Experiment

As the QTRAP 4000 results were so successful in accurately determining the PK properties of VSe1-28, the time course experiment was repeated with a biological triplicate per time point to provide a better statistical analysis of the variation present between biological samples. Unfortunately, the QTRAP 4000 was unavailable to repeat the experiment so the Agilent QTOF 6546 was used to acquire the results. The LC-MS method from the QTRAP 4000, “3011 Caf 18 min w MRM Q3” was transferred over to the QTOF 6546.

Preliminary investigations for VSe1-28 and caffeine were conducted on the QTOF 6546 to determine retention times and develop a calibration curve for time course experimental analysis. VSe1-28 was determined to have an RT of 1.886 min $\pm$ 0.3 min and caffeine an RT of 4.733 min $\pm$ 0.2 min as shown in figure 4.4. For the selection of concentration range a stock solution of VSe1-28 was used to determine the linear dynamic range of the instrument by serial dilution. This led to an excellent spectral resolution and good signal to noise ratio for the analytes of interest with a standard curve produced linear dynamic range of 0.005 μ M – 1 μ M, a strong linear fit ($R^2 = 0.99$) (figure 4.5) and an LOD of 10 fmol and an LOQ of 50 fmol was achieved. Unfortunately, due to an oversight, the biological samples spiked with a concentration of 42 μ M were not diluted prior to injection into the instrument and the time points with a concentration higher than 1 μ M could not be determined. For the purposes of this subchapter, the concentrations above the linear dynamic range of 1 μ M have been estimated using the linear regression formula generated for this experiment.

Figure 4.4 Extracted ion chromatograms overlayed to evaluate the retention times of VSe1-28 and caffeine at a concentration of 1 μM and 0.5 μM , respectively.

Figure 4.5 VSe1-28 normalized to 0.25 μM internal standard caffeine calibration curve performed on the QTOF 6546.

MS level scans were used for the quantitative analysis of VSe1-28 by determining the A.U.P. from each EIC for the biological and technical triplicates of m/z 281.0459. The A.U.P. was then averaged and used to build the PK figures conducted on the QTOF 6546 as seen in figures 4.6 and 4.7. such as half-life were concluded using the linear regression in figure 4.1 and it was determined that VSe1-28 had a half-life of 5.43 hours in lysate (1.24 μ M half-life concentration) (figure 4.7) and the half-life in growth media was determined to be 4.05 hours (15.23 μ M half-life concentration) (figure 4.6).

The normalized standard error was determined by calculating the technical and biological triplicates standard deviations and propagating the relative error by squaring then adding the relative standard errors of VSe1-28 and caffeine together, taking the square root and multiplying by the normalized average concentration. The lysate concentrations were subjected to a back-calculation dilution ($C_1V_1=C_2V_2$) to account for the 0.5 mL washing of hypotonic buffer before scraping transfer to a 1.5 mL centrifugation tube. The cumulative standard error of VSe1-28 did not exceed 7% in lysate and 2% in growth media.

It was observed that VSe1-28 is present in lysate and growth media up for an estimated ~20-hour period similar to the plasma stability study³¹. Both the lysate and media 24-hour time point were ascribed a concentration of zero as the EIC was unable to extract an observable concentration. This indicates that the concentration was either below the limit of detection of the instrument or had been completely depleted *in vitro*, the more plausible of the two being the former. Similar to the QTRAP results, a large concentration of VSe1-28 was found in the growth media plot (figure 4.7) and also did not enter the cells to a significant degree in the 15-minute time span. Contradicting the 67% remaining on the

QTRAP, an observed 41% of VSe1-28 was present after 3 hours which was comparable to the $38.9\% \pm 0.52$ found in the plasma stability study³¹.

When comparing the results from the QTRAP 4000 and the QTOF 6546 there are deviations between the single biological and triplicate sample analysis. Considering the growth media results, the half-life of VSe1-28 on the QTRAP 4000 and QTOF 6546 was determined to be 4.83 hours with a concentration of 19.55 μM and 4.05 hrs with a concentration of 15.23 μM , respectively. These results were deemed comparable to each other considering the rapid change in concentration within an exponential decay.

Considering the lysate results, the half-lives of VSe1-28 on the QTRAP 4000 and QTOF 6546 were determined to be 3.90 hrs with a concentration of 0.655 μM and 5.43 hrs with a concentration of 1.24 μM , respectively. A 1.5-hour difference in half-life is a significant difference, especially considering the exponential decay observations in concentration. To compare the QTRAP and QTOF half-life results, the half-life from the QTRAP (3.90 hrs) was inputted into the exponential decay equation of the QTOF time course results producing a concentration of 1.94 μM . When we compare these two values, 1.90 μM and 1.24 μM from the QTRAP and QTOF respectively we see that they are close in concentration indicating the possible validity of the plot of figure 4.6 indicating a successful experiment. Although the results were deemed successful for the QTOF biological triplicate, it is essential to repeat the time course experiment with the proper dilution to confirm the results on the QTOF 6546. More desirably, the time course experiment should be conducted on the QTRAP 4000 as the specificity and sensitivity allows for a more precise analysis of the results. This repetition will confirm the validity of the extrapolated results determined of the QTOF 6546.

Figure 4.6 VSe1-28 normalized to 0.25 μM internal standard caffeine monitored using MS level scans on the QTOF 6546 in lysate over a period of 24 hours.

Figure 4.7 VSe1-28 normalized to 0.25 μM internal standard caffeine monitored using MS level scans on the QTOF 6546 in growth media over a period of 24 hours.

4.2.1 QTOF 6546 Metabolites

Phase I and phase II metabolism are responsible for the biotransformation of foreign substances such as drugs in the body³⁵. Phase I metabolism is responsible for the transformation of parent compounds into more polar metabolites via cytochromes P450 enzymes which perform hydroxylations^{35,84}. Phase II enzymes are responsible for the transformation of parent compounds into metabolically inactive compounds and subsequent conjugation with the inactive compound to make an excretable compounds by increasing their hydrophilicity³⁵. Of interest to our group is glutathione as it has been previously demonstrated that VSe1-28 reacts with free glutathione in cells^{18,31}. The tripeptide, γ -L-glutamyl-L-cysteinyl-glycine (GSH), amino acid sequence (ECG), is derived from the reduction of glutathione disulfide (GSSG) by the coenzyme NADPH. GSH is found in the cytosol of cells in concentrations of 1-2 mM while outside the cell in a micromolar range^{34,85}. As such, it is important to consider whether GSH-VSe1-28 adduct is found within our biological samples both in the lysate and growth media. The phase II enzyme glutathione S-transferase P (GSTP1-1) is responsible for the catalyzation through deprotonation of glutathione which in turn conjugates to electrophiles for the purpose of detoxifying, and secreting xenobiotics from the cell⁸⁶. This reaction, also known as a Michael addition reaction, occurs through the nucleophilic addition of the thiol to an α , β -unsaturated carbonyl⁸⁷ present in VSe1-28 and shown in figure 4.9.

Figure 4.8 Michael addition mechanism of the addition of VSe1-28 to Glutathione.

It has been previously observed that GSH levels are depleted for a minimum of 4 hours when cell cultures are treated with VSe1-28¹⁸. Furthermore, it was determined that although GSH does form an adduct with VSe1-28 it is not a contributing factor in the antiviral effects of the drug therefore the monitoring of the adduct is for degradation purposes. It is also known through previous plasma stability studies that GSTP1-1 quickly degrades some VSe analogues³², VSe1-28 having one of the longer observed GSH half-life of 96 minutes with 38.9% $\pm$ 5.2 remaining compound in solution after 3 hours (See Appendix A).

A crude GSH-VSe1-38 adduct was extracted and purified from a cell culture to investigate the precursor and product ions. As shown in figure 4.10, the precursor ion GSH-VSe1-28 adduct (EC(3011)G) was observed with the expected single chlorine isotope pattern with a m/z 552.1051 which was comparable to the expected m/z 552.1531. Unbound GSH was also observed with a m/z 308.0846 and was also comparable to the expected m/z 308.0916. GSH-VSe1-28 adduct was subsequently subjected to a product ion scan with a collision energy of 25 eV which revealed EC(3011)G with the loss of water (m/z 534.1275) and Cysteinylglycine (CysGly) bound VSe1-28 (C(3011)G, m/z 423.1016). Since C(3011)G was found in the full scan it is possible that this is not a product ion of EC(3011)G however it was kept at as m/z of interest as a cell surface enzyme, gamma-glutamyl transpeptidase (GGT) is known to cleave glutamine residues off GSH⁸⁸.

Figure 4.9 Crude extract of a GSH adduct bound to VSe1-28 (m/z 552.1051), GSH (m/z 308.0846) and GSH-VSe1-28 -E (m/z 423.1015).

Figure 4.10 MS/MS of the GSH-VSe1-28 adduct (m/z 552.1051) with the formation of product ion C(3011)G (m/z 423.1016).

From the observed results present in the product ion scans, GSH (m/z 308.0846), GSH-VSe1-28 (m/z 552.1051) and CysGly-VSe1-28 (m/z 423.1016) adducts were deemed of interest in the search for metabolites of interest in the time course experiments (figure 4.11). The GSH-VSe1-28 adduct was extracted first and produced the results shown in figures 4.12 and 4.13. The MS level scans were used in extracting the A.U.P. firstly for the GSH-VSe1-28 adduct was extracted for all biological triplicate time course points and plotted over 24 hours. Interestingly, the results of figure 4.15 demonstrate that free GSH in lysate is immediately conjugated to VSe1-28 while in growth media GSH-VSe1-28 adduct rises slightly over time before being depleted.

Figure 4.11 GSH (m/z 308.0846) (a), GSH bound to VSe1-28 (m/z 552.1051) (b) and GSH-VSe1-28 metabolite (m/z 423.1016) (c).

Figure 4.12 Extracted ion chromatogram of the GSH-VSe1-28 adduct (m/z 552.1533) in lysate over a period of 24 hours.

Figure 4.13 Extracted ion chromatogram of the GSH-VSe1-28 adduct (m/z 552.1533) in growth media over a period of 24 hours.

Figure 4.144 Averaged area-under-the-peak (A.U.P.) from the extracted ion chromatograms (EICs) of the biological triplicate of GSH-VSe1-28 adduct pharmacokinetic analysis over a period of 24 hours in lysate.

Figure 4.15 Averaged area-under-the-peak (A.U.P.) from the extracted ion chromatograms (EICs) of the biological triplicate of GSH-VSe1-28 adduct pharmacokinetic analysis over a period of 24 hours in growth media.

The GSH-VSe1-28 – E adduct was observed as multiple extracted peaks in both the lysate and growth media as shown in figures 4.16 and 4.17. CysGly is produced by the cell surface enzyme, gamma-glutamyl transpeptidase (GGT) which is known to cleave glutamine residues off GSH⁸⁸. CysGly is a metabolic breakdown product of GSH and could possibly be a by-product occurring following conjugation of GSH to VSe1-28. In a study, researchers attempted to define whether CysGly had an active role in the homeostasis of GSH in bovine eye lenses. The work demonstrated that CysGly possibly interferes with cysteine homeostasis and GSH biosynthesis. A key observation was noted that CysGly, a membrane product of GGT could intervene with the function of GSH therefore driving an efflux of GSH. The study concluded that the blocking of the GGT cycle elicits a 1.5-fold increase extra-cellular accumulation of glutathione⁸⁹.

Upon literature review there are a couple reasons for having multiple peaks and matching MS/MS spectra. Plausible reasons for why there are multiple peaks eluting with the same product ion could be due to an inadequate gradient suited for retaining, separating and resolving the CysGly adducts. Modifications to the gradient such as a slower increase in solvent %B could condense the peaks. As there are multiple peaks in both the growth media and lysate, matrix composition has been eliminated as suspect.

As described by Neta et al. certain product ions in electrospray ionization tandem MS can react with residual water in the collision cell⁹⁰. Interestingly, the study identified in various drugs, pesticides, metabolites, and other compounds dissolved in water with F.A. that the product ions incurred a loss of glutamate and the addition of water. Compounds such as folic acid, methopterin, raltitrexed and others were observed to lose glutamate upon MS/MS fragmentation and ensuing reaction with water leading to a $[M+H - \text{Glu} + \text{H}_2\text{O}]^+$

ion. Although the study focuses on MRM results in terms of loss of quantitation, it is still applicable to our work as we have identified possible artifacts which can be described by this work. As this unavoidable reaction is possible it is important to consider this observation if building an MRM method for GSH-VSe adducts.

Figure 4.16 Extracted ion chromatograms of the cysteinylglycine-VSe1-28 adduct (m/z 423.1103) in growth media over a period of 24 hours.

Figure 4.17 Extracted ion chromatograms of the cysteinylglycine-VSe1-28 adduct (m/z 423.1103) in lysate over a period of 24 hours.

Furthermore, to demonstrate that the peaks were all from the same precursor, CysGly adducts from lysate and growth media were plotted to analyze the trends of each peak. If the trends between peak times differed when plotted, this could indicate a different metabolite. The peaks in the lysate CysGly-VSe1-28 adducts that were plotted included, 0.71 min, 1.56 min and 2.14 min as shown in figure 4.18. Although there were more peaks, these three were selected for their peak presence throughout the full 24 hours. The 0.71 min and 2.14 min resembled the same trend observed in the lysate GSH-VSe1-28 adduct (figure 4.15) and indicates similarities between the two. Although the 0.156 min peak resembles the growth media pattern, there is a chromatographic shift of the peak which could be attributing to A.U.P. differences between the 0.25 hr peak and the 4 hr peak. CysGly rapid depletion in lysate following VSe1-28 treatment is expected and the observed increase in metabolite at the 8-hour mark directly correlates with previous studies³¹.

The growth media CysGly-VSe1-28 plot shown in figure 4.19 follows the trend shown in figure 4.16 where the 0.75 min, 1.56 min, 2.14 min and 3.05 min all show a sharp increase in metabolite followed by a slow decrease. The sharp increase can be attributed to the rapid accumulation of conjugated CysGly-VSe1-28 followed by rapid depletion of CysGly. In summary, the GSH-VSe1-28 and CysGly-VSe1-28 peaks have a similar trend in metabolite increase indicating a direct correlation between the two. This correlation could mostly likely be due to the formation of GSH-VSe1-28 and subsequent breakdown intra and extra-cellularly. It could also represent the cleavage of GSH by the cell surface enzyme gamma glutamyl transferase.

Figure 4.18 Averaged area-under-the-peak (A.U.P.) from the extracted ion chromatograms (EICs) of the biological triplicate of where 0.71 min, 1.56 min and 2.14 min represent the four most prevalent extracted peak A.U.P.'s present in figure 4.17 of-VSe1-28 adduct (m/z 423.1533) over a period of 24 hours in lysate.

Figure 4.19 Averaged area-under-the-peak (A.U.P.) from the extracted ion chromatograms (EICs) of the biological triplicate of where 0.75 min, 1.56 min, 2.14 min and 3.05 min represent the four most prevalent extracted peak A.U.P.'s present in figure 4.16 of CysGly-VSe1-28 adduct (m/z 423.1533) over a period of 24 hours in growth media.

The precursor C(3011)G m/z 423.1106 which produced the multiple peaks in the EICs of lysate and growth media was subjected to auto MS/MS during analysis and produced the same product ion spectrum for all peaks. The example shown in figure 4.20 and figure 4.21 exemplifies the common ions that were observed in the MS and MS/MS level scans which were also observed in figure 4.10. Ions such as m/z 405.2317, m/z 383.0478 were found in both crude and time course experiments. The structural identification of these peaks is still unknown however could indicate possible GSH breakdown products not previously acknowledged in this work.

Figure 4.20 MS level scan of CsyGly-VSe1-28 adduct (m/z 423.1103) in growth media at 8 hours.

Figure 4.21 MS/MS of CysGly-VSe1-28 adduct -E (m/z 423.1103) of the 1.5-minute peak in growth media at 8 hours.

Subsequent searches determined that GSH and GSSG were either not present or the concentrations were below the limit of detection of the instrument within lysate and growth media negative controls as shown in figures 4.22 and 4.23 (See Appendix B and C for GSSG). The presence of GSH and GSSG should be found in lysate and, therefore, is assumed that it is below the limit of detection of the QTOF for lysate and growth media. If tandem MS had been applied to search for GSH or GSSG the sensitivity could have been increased as studies have demonstrated a detection level of 500 amol using a QTRAP 4000⁸⁵. Some *in vitro* cell culture medias do contain glutathione such as M199, McCoy's 5A Modified Medium, and Waymouth Medium MB. OptiPro SFM however does not contain any added glutathione and would most likely exist in its oxidized (GSSG) form in serum free media. As stated in the documentation for OptiPro SFM (ThermoFisher), it is a serum-free, protein-free, and animal origin free media⁹¹ therefore added GSH and GSSG would not be present indicating Vero cells are responsible for any extracellular oxidized or reduced glutathione.

Figure 4.22 Extracted ion chromatogram of GSH (m/z 308.0916) in lysate over a period of 24 hours which was deemed not present.

Figure 4.23 Extracted ion chromatogram of GSH (m/z 308.0916) in growth media over a period of 24 hours which was deemed not present.

Further investigations are required to elucidate the multiple peak identifications and product ions of GSH-VSe1-28 – E. With the retention times of each GSH-VSe1-28 – E peak known; the time course experiment can be repeated using an isotopically labelled version of VSe1-28 to confirm the metabolite's structural identity. Not only is the application of isotopically labelled versions of potential drug candidates beneficial to metabolic studies in MS-based analysis but also in NMR where isotopic ratio precision of structure-specific analytes can be resolved^{92,92}. If a metabolite is extracted from the HPLC process it can be analyzed by NMR techniques which have been proven to provide reliable and rigorous metabolite profiling of crude biological extracts⁹².

Future metabolites identified *in vivo* or *in vitro* can be determined through this isotopic labelling process and has been prepared by collaborators at the university of Ottawa. Chemists at the university of Ottawa have prepared an isotopically labeled version of VSe1-28 by replacing 2 x ¹³C and 1 x ¹⁵N increasing the mass by +5 Th as shown in figure 4.24. An added advantage to the metabolomics analysis of VSe1-28 is the distinct chlorine isotope pattern not abundantly present in naturally occurring *in vivo* cultures. Recently developed software's continue to evolve in their abilities to analyze stable isotope-labeled metabolomics data⁹³. With the combination of standard and isotopically labeled versions of drug candidates, LC-MS is capable of elucidating metabolites and their structural identity.

Figure 4.24 Heavy isotopically labelled VSe1-28 with two ^{13}C atoms and one ^{15}N (m/z 287.07309).

4.3 Conclusions

It has been found that when Vero cells are treated with VSe1-28 at a concentration of 42 μM and monitored over a period of 24 hours, the half-life was found to be 5.43 hours in lysate and 4.05 hours in growth media on the QTRAP 4000.

The extrapolated results achieved on the QTOF 6546, following the same treatment of Vero cells with VSe1-28 with 42 μM and monitored over a period of 24 hours, found the half-life to be 3.90 hours in lysate and 4.83 hours in growth media. To confirm the results from the limited linear dynamic range of the QTOF 6546 calibration curve, the biological triplicate time course experiment will need to be repeated and diluted a minimum of 42x prior to injection. Preferably, if access to a TQMS is available, this experiment should be conducted on this instrument for a higher specificity and selectivity of VSe1-28. In addition, the time course experiment should be repeated a third time using the heavy isotopically substituted VSe1-28 to further confirm the metabolites identified in regular VSe1-28 version.

The results of the QTOF 6546 demonstrated the rapid conjugation of VSe1-28 to GSH and was found to rapidly deplete the concentration of GSH found within cells. Cysteinylglycine was also identified as a metabolite however further investigations need to be done with respect to the multiple peaks found within the EICs. Subsequent searches determined that the concentration of GSH and GSSG were below the limit of detection of the instrument within lysate and growth media.

Chapter 5: Proteomics

5.1 Porcine Hemoglobin Study

The majority of drug target identification and characterization begins with attempting to identify the interacting target (proteins/genes). To reach clinical phase I trials, the viral sensitizers need to be proven to be efficacious, safe and have a reasonable mode of delivery. To achieve this, multiple tools are available for target identification such as cell-based models, protein interactions, and signaling pathways. Following target identification, target validation must demonstrate the supposed functionality. In the following study, we attempt to prove the binding location of VSe1-28 to Cys38 in the DNA binding pocket of p65. To accomplish this, the short peptide sequence YKCEGR was synthesized to develop MRMs and target specific MS/MS methods to validate the molecular target of VSe1-28 as discussed in chapter 3. Later discovered in chapter 3, iodoacetamide was replacing VSe1-28 on the cysteine of YKCEGR during a standard tryptic digestion, which lead to the simplification of the tryptic digestion protocol. As such, the YKC(Alk)EGR quantitative and qualitative transitions were no longer considered for experiments in chapter 5.

The identification of proteins by MS is the most critical step in proteomic studies by either peptide mass fingerprinting (PMF) or amino acid sequencing of peptides. PMF-based studies use proteolytic cleavage to break down proteins into a group of peptides which provide a unique “fingerprint” of the protein⁹⁴. Unique peptide collections can be analyzed by LC-MS and compared against theoretical peptide masses of proteins in databases such as NCBI or Swiss-Prot⁹¹. Database search engines are an invaluable tool in

modern methods of annotating spectra with peptide sequences⁹². The following *in vivo* and *in vitro* p65 digestion studies used MASCOT (Matrixscience).

The p65 *in vivo* and *in vitro* samples contained limited amounts of product for analysis, with 130 pmol in the three modified *in vivo* samples and 46 pmol in the three modified *in vitro* modified samples with VSe1-28, DMF and, DMSO. Before conducting the investigations on limited amount of sample, an LC-MS method was developed for the QTOF 6546 for proteomics work. The QTOF 6546 allows for two types of MS/MS options for analysis: Auto MS/MS and Targeted MS/MS. Auto MS/MS is a method which conducts MS/MS on ions which match the parameters specified in the method parameters. For instance, Auto MS/MS can be modified to only trigger MS/MS upon identification of ions with a charged state of +2, all other ions would only be measured at MS level. Targeted MS/MS is the second option which allows for a more m/z specific MS/MS triggered analysis. An example would be setting the parameters of the targeted MS/MS method to scan for a m/z of 205 at a retention time of 5.1 min $\pm$ 0.2 min. Targeted MS/MS would be a more desirable option for this type of analysis however important parameters were required for proper triggering of MS/MS such as the retention time of m/z and Δ RT. With the limited volume of *in vivo* and *in vitro* samples and the requirement to inject once prior to analysis to assess the retention times, targeted MS/MS was rejected as the MS/MS option of choice. As such, Auto MS/MS was selected as the retention times of important masses could not be determined prior to analysis.

The injection volume was reduced from 20 μ L to 10 μ L to optimize sample use while still injecting enough sample for MASCOT identification. The LC gradient method “digested peptides” shown in figure 2.3 was considered suboptimal for proteomics work and alternative gradients were considered. The optimized gradient developed for proteomics on the Agilent 6546 was based on an application note provided by Agilent⁹³ which elongated the digested peptide gradient by 5 minutes from 2% B to 100% B. The longer gradient provided a slower composition change from solvent A to solvent B which enhanced the separation of peptides.

Following LC optimization and selection of MS/MS method, 5 mg of lyophilized hemoglobin porcine (Sigma Aldrich) was diluted and vortexed in 20 mL of HPLC grade 0.1% F.A. to make-up a 250 μ g/mL stock solution. This solution was then used for a serial dilution of 100 ppm, 50 ppm, 25 ppm, 10 ppm, 5 ppm and 1ppm used for LC-MS optimization. The most successful MS method for proteomics was developed as a result of the combination of suggested parameters from Agilent applications notes⁷⁵ and single parameter modification experiments. The mass scan range was set to 100-1600 Da for optimal peptide detection. Next, the abundance-dependent accumulation parameter was reduced to a target of 15,000 counts/spectrum rather than the pre-selected 20,000 counts/spectrum. This allowed for the triggering of Auto MS/MS to occur at a lower count with the intention of capturing lower abundance ions present in a potentially low concentration of the modified p65 samples. To ensure only abundant peptide masses were targeted with the reduced target counts to 15,000 counts/spectrum, a static exclusion range table was added between 100-250 Da to reduce unwanted analysis on insignificant masses. Other important parameters that were set included setting the isotope model to peptides

with the QTOF sorting precursors by abundance and with a precursor charge-state preference for charges of +2, +3 and $> +3$, excluding +1. The best LC-MS method as determined by the best sequence coverage and MS/MS coverage was named HG_NG_Test1, hemoglobin (HG) new gradient (NG), test 1 being the first of various QTOF parameter tests. This method determined an 80% sequence coverage of a 100ppm digested hemoglobin porcine as shown in figure 5.1.

It was also determined using the HG_NG_Test1 method and the 10uL injection that the LC-MS method could detect 3 pmol (5 ppm) of hemoglobin porcine digest with a sequence coverage of 23%. Below 3 pmol, the 0.619 pmol (1ppm) sample was not identified via MASCOT analysis indicating the sample concentration was too low to produce adequate MS/MS analysis for MASCOT identification. This study demonstrated that the simplified tryptic digestion protocol worked effectively, and the mass of protein injected as low as 5 ppm of hemoglobin was sufficient for MASCOT identification purposes.

Protein View: HBB_PIG

Hemoglobin subunit beta OS=Sus scrofa OX=9823 GN=HBB PE=1 SV=3

Database: SwissProt
Score: 229
Monoisotopic mass (M_r): 16212
Calculated pI: 7.10
Taxonomy: Sus scrofa

Protein sequence coverage: 80%

Matched peptides shown in **bold red**.

1 MVHLSAEEKE **AVLGLWGKVN VDEVGGEALG RLLVVYPWTQ RFFESFGDLS**
51 **NADAVMGNPK** VKAHGKKVLQ SFSDGLKHLN NLKGTFAKLS ELHCDQLHVD
101 **PENFRLLGNV IVVVLARRLG HDFNPNVQAA FQKVVAGVAN ALAHK**YH

Figure 5.1 MASCOT results providing an 80% sequence coverage of hemoglobin porcine at a concentration of 50 ppm using an optimized proteomics QTOF LC-MS method.

5.2 Mechanism of action identification for *in vivo* and *in vitro* modified p65 protein

Similar to the metabolomics experimental design, DMSO was added in a parallel cell treatment time course trial to compare the negative control to the results of the time course VSe1-28 experiments. Metabolites found in the VSe1-28 treated samples but not in the DMSO samples were considered a primary method of positively identifying true metabolites. For the MOA experiment, DMSO was also used as a negative control, in addition DMF was added as a positive control to identify the MOA of VSe1-28. The drug DMF has been well studied as an inhibitor of NF- κ B p65, its MOA is well understood where it and VSe share an α , β unsaturated carbonyl moiety^{20,31}. As such, DMF was added for observation bound to YKCEGR (YKC(DMF)EGR, m/z 899.3932). Successful treatment of *in vivo* and *in vitro* experiments with DMF would demonstrate that the experiment was producing proper results and therefore should be seen in the VSE1-28 treated samples. For this reason, table 5.1 was developed to extract EICs of the three most expected peptides and digested products.

Table 5.1 List of m/z YKCEGR, modified negative control YKC(DMF)EGR, YKC(3011)EGR and digested products for analysis.

<i>Peptide</i>	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
YKCEGR	755.3505	378.1789	252.4550
YKC(3011)EGR	999.4124	500.2101	333.8093333
YKC(DMF)EGR	899.3932	450.2005	300.4696
CEGR	464.1922	232.5997	---
C(3011)EGR	708.2536	354.6307	---
C(DMF)EGR	608.2344	304.6211	---

For a successful PMF-based protein identification a robust MS/MS method is necessary in identifying low concentrations of peptides, providing an extensive file to compare against large peptide databases⁹¹. This robust method was produced in section 5.1 and deemed a reliable proteomics method for these experiments. The p65 modified samples were prepared for analysis by diluting the lyophilized digested proteins in water with 0.1% F.A. A 10 μ L injection of each of the digested *in vitro* and *in vivo* p65 modified VSe1-28, DMF and DMSO was conducted and produced the TICs of figures 5.2 and 5.3. The observed TICs indicated that MS/MS was not triggered. Next, EICs of the expected peptides from table 5.1 were extracted and did not identify any masses of interest. It can be reasonably assumed that based on the nature and abundance of p65 present *in vivo* that these modified samples could potentially have a lower amount present than *in vitro* samples however both samples are observed to have minimal peptide presence. With this observation plausible reasons for loss of protein or peptide were considered.

Figure 5.2 TICs of digested *in vivo* p65 modified with VSe1-28 (a), DMF (b), or DMSO (c) injected onto the Agilent 6546 using the Auto MS/MS method, HG_NG_Test1 method.

Figure 5.3 TICs of digested *in vitro* p65 modified with VSe1-28 (a), DMF (b), or DMSO (c) injected onto the Agilent 6546 using the Auto MS/MS method, HG_NG_Test1 method.

The unsuccessful attempts at positively identifying the mechanism of action of VSe1-28 lead to a critical evaluation of the sources of error within the experimental design. The protocol for the extraction, modification, and purification process of p65 was evaluated and deemed satisfactory. The next possible source of error was the digestion protocol outlined in chapter 2, subsection 2.4.3. and 2.5.4. The S-TRAP microcolumns function by allowing denatured, non-digested proteins to bind to the S-TRAP column (Profiti) via centrifugation. The column is composed of derivatized silica allowing for multiple weak-affinity interactions with undigested protein. The micron sized pore composition of the derivatized silica allows for maximum surface area for washing and rapid tryptic digestion. As the trap does not have affinity towards peptides, they elute after digestion⁷². Within the digestion protocol provided by Profiti, reduction and alkylation are the third step of a 14-step process. It is plausible that without reduction and alkylation the proteins are much larger in size and the enzyme digestion process may not be as efficient within the micro pores. Upon trypsin addition the spin column is capped and allowed to incubate for 1 hour at 47 °C. This vastly differs from a standard tryptic digestion protocol which allows for overnight incubation to ensure complete digestion. As a result of the removal of step 3, the proteins may not perform as expected by the marketed 1-hour rapid digestion process.

As an extensive hemoglobin digest study was performed and various concentrations were analyzed on the QTOF it was an excellent control to use and compare the S-TRAP method versus a trypsin only in-house digestion. To validate this suggested modified tryptic digestion protocol, 100 µg (50 ppm) of hemoglobin was dried down and sent to OHRI for digestion using the S-TRAP protocol. Upon return, hemoglobin fraction 2 (80 µL of 0.2% F.A.) was combined with in the lyophilized fraction 3 tube. The tube was dried

down under a flow of nitrogen and resuspended in 200 μ L of HPLC grade H₂O and 0.1% F.A. Assuming complete digestion and elution from the S-TRAP column there should be 100 μ g of HG in the 200 μ L solution (500 ppm) of OHRI digested HG. The solution was then used as a stock to prepare a 100 ppm and 50 ppm HG solution to evaluate MASCOT's sequencing scores and digestion efficiency. In theory, the results of the two digests should have similar digestion efficiencies and MASCOT scores however it was important to consider that the digestion efficiency on the S-TRAP column might not elute 100% of the protein digested and possibly much smaller amounts of peptide were eluted. When assuming even a 50% digestion efficiency and elution, the concentration would be 250 ppm and contain 154 pmol of peptides per 10 μ L injection. Upon digestion, transfer back to Carleton university over dry ice and injection onto the instrument a large decrease in Auto MS/MS coverage was observed. This discovery identified the source of the low sequence coverage of the in vitro and in vivo p65 modified samples.

Figure 5.4 OHRI digest using S-TRAP protocol in subsection 2.4.3 using 100 μ g of dried down hemoglobin (a). A 5ppm hemoglobin digest completed in-house (b).

A modified tryptic digest protocol was developed following the identification of a possible source of error in the digestion and elution efficiencies of the S-TRAP microcolumns. The protein would remain in the same Eppendorf post column elution as described in chapter 2 subsection 2.5.2. As the proteins in both the *in vivo* and *in vitro* S-TRAP digestion were both eluted using a S-TRAP buffer, only a small amount of ABC was added to each tube to compensate for buffer already present. Proteins were then denatured by heating at 70°C for 5 minutes on a heated shaker at 200rpm. Sequencing-grade trypsin (Promega) was re-constituted into a 50 mM ammonium bicarbonate solution and was added to each protein in a 1:20 (w/w; protease: protein) ratio. The proteins were incubated over night at 37°C. This simplification would prevent loss of protein between transfer to the S-TRAP column and incomplete digestion on column.

A final attempt was performed using old p65 modified *in vivo* samples from 6 months prior which were not subjected to digestion. to positively identify p65 protein and/or p65 modified samples by performing a simple trypsin digest in an Eppendorf. Fortuitously, the p65 protein was positively identified in all three modified samples, with a sequence coverage of 2%, 20%, and 25% for VSe1-28, DMF, and DMSO respectively (See Appendix D and E). Although the VSe1-28 sample had only a 2% sequence coverage, it can be assumed another injection could provide an enhanced sequence coverage.

Protein View: TF65_HUMAN

Transcription factor p65 OS=Homo sapiens OX=9606 GN=RELA PE=1 SV=2

Database: SwissProt
 Score: 162
 Monoisotopic mass (M_r): 60181
 Calculated pI: 5.46
 Taxonomy: Homo sapiens

Protein sequence coverage: 25%

Matched peptides shown in **bold red**.

1	MDELFLIFP	AEPAQASGPY	VEIIEQPKQR	GMRFRYKCEG	RSAGSIPGER
51	STDTTKTHPT	IKINGYTGP	TVRISLVTKD	PPHRPHPHEL	VGKDCRDGFY
101	EAELCPDRCI	HSFQNLGIQC	VKKRDLEQAI	SQR IQTNNNP	FQVPIEEQRG
151	DYDLNAVRLC	FQVTVRDPSP	RPLRL LPPVLS	HPIFDNRAPN	TAEIKICRVN
201	RNSGSCLGSD	EIFLLCDKVQ	KEDIEVYFTG	PGWEARGSFS	QADVHRQVAI
251	VFR TPPYADP	SLQAPVRVSM	QLRRPSDREL	SEPMEFQYLP	DTDDRHRIEE
301	KRKRTYETFK	SIMK KSPFSG	PTDPRPPRR	IAVPSRSSAS	VPKPAPQPYF
351	FTSSLSTINY	DEFPTMVFP	GQISQASALA	PAPFQVLPQA	PAPAPAPAMV
401	SALAQAPAPV	PVLAPGPPQA	VAPPAPKPTQ	AGEGTLSEAL	LQLQFDDDEL
451	GALLGNSTDP	AVFTDLASVD	NSEFQQLLNQ	GIPVAPHTTE	PMLMEYPEAI
501	TRLVTGAQRP	PDPAPAPLGA	PGLPNGLLSG	DEDFSSIADM	DFSALLSQIS
551	S				

Figure 5.5 Positive identification of p65 from extracted *in vivo* negative control p65 DMSO sample using only trypsin.

Although the MS-based *in vivo* and *in vitro* experiments were unsuccessful in identifying p65 as the molecular target of VSe1-28, work at our collaborative institutes, OHRI and university of Ottawa were successful in performing a competitive activity-based protein profiling experiment using DMF and VSe1-28³³. As it was previously known that DMF covalently binds to p65, VSe1-28 was also assumed to target the same location¹⁷. Under this assumption, cells were pre-treated with VSe1-28, DMF or a negative probe then with the addition of DMF. The experimental results following a biotin azide pulldown resulted in the detection of DMF bound to p65³³ in the DMF and negative probe while the VSe1-28 pulldown did not yield the same level of detection. This exemplified that VSe1-28 can compete for p65 binding like DMF. These results demonstrated that VSe1-28 covalently modifies NF- κ B p65.

5.3 Conclusions

In summary, a proteomics HPLC-QTOF-MS/MS method was developed and optimized through a standard hemoglobin porcine digestion study. This study determined that the LOD of the instrument with respect to hemoglobin peptides was 3 pmol. With this information, the *in vitro* and *in vivo* modified p65 samples were resuspended in a volume of HPLC grade 0.1% F.A. that would ensure a detectable concentration above the LOD when injected onto the QTOF.

Unfortunately, the low protein concentration in the *in vivo* and *in vitro* samples led to an insufficient concentration for LC-MS/MS analysis. It can be postulated based on the results of the trypsin digestion of modified *in vivo* p65 samples that the S-TRAP column is a possible culprit in reducing the peptide elution amount. As a result, the proteomics experiments were unsuccessful in confirming the mechanism of action of VSe1-28 with p65 however switching to the simplified protocol could allow for a successful future determination.

A timely simplified digestion protocol was conducted on old *in vivo* p65 modified samples and was successful in identifying p65 in all three treated lines, VSe1-28, DMF and DMSO. With the achieved results, it has been demonstrated that a simplified protocol prevents the loss the peptide between multiple transfers and potential issues with the S-TRAP columns.

Chapter 6: Concluding Remarks

Oncolytic virotherapies can provide a powerful targeted alternative to traditional cancer therapies. The combination of VSVs with a viral sensitizer can overcome the limitations caused by heterogeneity in cancerous cells, providing an enhanced combination therapeutic treatment for cancer. LC-MS techniques have been applied to monitor the pharmacokinetics and metabolomic activity of our viral sensitizer, VSe1-28 with emphasis on the metabolic activity in Vero cells over a period of 24 hours. Glutathione was subsequently found in the time course experiment as a main metabolite. Future work in repeating the time course experiment could incorporate the isotopically labelled VSe1-28 in cell cultures to positively identify ions as metabolites and later structural determination.

Proteomics-based studies were performed where a proteomics LC-QTOF-MS method was developed through a standard hemoglobin digest study. Following optimization of a proteomics method, *in vivo* and *in vitro* experiments of p65 modified protein with VSe1-28, DMF (positive control), and DMSO (negative control) were conducted to support previous MOA findings^{18,33} however approaches were unable to positively identify the VSe1-28 MOA. Although the modified protein was not positively identified, MASCOT was able to identify p65 through a simplified digestion method. This modified protocol described in this dissertation will promisingly provide proof of the MOA of VSe1-28.

Appendix

Appendix A Table ranking the VSe analogues in order of highest (green) to lowest (red) plasma stability³².

Table 4.5: Ranking the VSe analogues in order of highest (green) to lowest (red) plasma stability

Structure (or amine R-group)	Compound	Plasma Stability (% remaining at 3 hrs)	Activity (Normalized to Compound 1)
20	102.95	0
21	102.66	0
16	98.19	0
24	91.64	0.34
8	88.29	0
17	82.01	0
22	71.97	0.06
14	70.24	0.03
3	65.66	0
26	64.8	0.26
35	63.81	0.02
43	58.32	0.15

29	57.6	0.39
25	54.83	0.53
45	54.22	0.01
52	54.12	0.03
41	51.43	0.22
53	50.1	0.06
42	49.11	0.01
12	47.64	0
44	45.85	0.15
49	44.62	0.04
51	43.97	0.05
30	42.9	0.61
11	42.47	0

39	41.38	0.06
31	40.08	0.34
50	39.61	0.08
28	38.92	0.67
48	36.46	0.03
36	28.23	0.04
38	25.74	0.06
46	23.12	0.17
47	22.74	0.1
10	19.81	0.3
40	15.31	1.6
15	14.87	0

27	8.96	0.04
37	0.66	0.06
33	0	0.02
13	0	0
9	0	0.49
7	0	0.07
6	0	0.31
5	0	0.47
4	0	0
2	0	0.47
1	0	1

Appendix B Extracted ion chromatogram of GSSG (m/z 613.1597) in lysate over a period of 24 hours which was deemed not present.

Appendix C Extracted ion chromatogram of GSSG (m/z 613.1597) in growth media over a period of 24 hours which was deemed not present.

Appendix D Positive identification of p65 from extracted *in vivo* positive control p65 DMF sample using only trypsin.

MASCOT Search Results

Protein View: TF65_HUMAN

Transcription factor p65 OS=Homo sapiens OX=9606 GN=RELA PE=1 SV=2

Database: SwissProt
 Score: 223
 Monoisotopic mass (M_r): 60181
 Calculated pI: 5.46
 Taxonomy: [Homo sapiens](#)

Sequence similarity is available as [an NCBI BLAST search of TF65_HUMAN against nr.](#)

Search parameters

MS data file: p65 dmf only trypsin.mgf
 Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
 Variable modifications: [Oxidation \(M\)](#)

Protein sequence coverage: 20%

Matched peptides shown in **bold red**.

```

1  MDELFLIFP AEPAQASGPY VEIIEQPKQR GMRFRYKCEG RSAGSIPGER
51  STDITKTHPT IKINGYTGPQ TVRISLVTKD PPHRPHPEL VGKDCRDGFY
101 EAEELCPDRCI HSFQNLGIQC VKKRDLEQAI SQRIQTNNNP FQVPIEEQRG
151 DYDLNAVRLC FQVTVRDPSG RPLRLPPVLS HPIFDNRAPN TAEIKICRVN
201 RNSGSCLGSD EIFLLCDKVQ KEDIEVYFTG PGWEARGSFS QADVHRQVAI
251 VFRTPPYADP SLQAPVRVSM QLRRPSDREL SEPMEFQYLP DTDDRHRIEE
301 KKKRTYETFK SIMKKSPFSG PTDPRPPRRR IAVPSRSSAS VPKPAPQYPY
351 FTSSLSTINY DEFTMVVFPQ GQISQASALA PAPPQVLPQA PAPAPAPAMV
401 SALAQAPAPV PVLAPGPPQA VAPPAPKPTQ AGEGLTSEAL LQLQFDDIDL
451 GALLGNSTDP AVFTDLASVD NSEFQQLLNQ GIPVAPHTTE PMLMEYPEAI
501 TRLVTGAQRP PDPAPAPLGA PGLPNGLLSG DEDFSSIADM DFSALLSQIS
551 S
  
```

Appendix E Positive identification of p65 from extracted *in vivo* VSe1-28 treated p65 sample using only trypsin.

MASCOT Search Results

Protein View: TF65_HUMAN

Transcription factor p65 OS=Homo sapiens OX=9606 GN=RELA PE=1 SV=2

Database: SwissProt
Score: 42
Monoisotopic mass (M_r): 60181
Calculated pI: 5.46
Taxonomy: Homo sapiens

Sequence similarity is available as [an NCBI BLAST search of TF65_HUMAN against nr.](#)

Search parameters

MS data file: p65 3011 only trypsin.mgf
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Variable modifications: Oxidation (M).

Protein sequence coverage: 2%

Matched peptides shown in **bold red**.

```
1  MDELFLIIFP AEPAQASGPY VEIIEQPKQR GMRFYKCEG RSAGSIPGER
51  STDTTKTHPT IKINGYTGPG TVRISLVTKD PPHRPHPEL VGKDCRDGFY
101 EAELCPDRCI HSFQNLGIQC VKKRDLEQAI SQRIQTNNNP FQVPIEEQRG
151 DYDLNAVRLC FQVTVRDPG RPLRLPPVLS HPIFDNRAPN TAEIKICRVN
201 RNSGSCLGGD EIFLLCDKVQ KEDIEVYFTG PGWEARGSFS QADVHRQVAI
251 VFRTPPYADP SLQAPVRVSM QLRPSDREL SEPMEFYLP DTDDRHRIEE
301 KRKRTYETFK SIMKKSPFSG PTDPRPPRR IAVPSRSSAS VPKPAPQPYP
351 FTSSLSTINY DEFPTMVFPs GQISQASALA PAPPQVLPQA PAPAPAPAMV
401 SALAQAPAPV PVLAPGPPQA VAPPAPKPTQ AGEGLTSEAL LQLQFDDDL
451 GALLGNSTDP AVFTDLASVD NSEFQQLNQ GIPVAPHTTE PMLMEYPEAI
501 TRLVTGAQRP PDPAPAPLGA PGLPNGLLSG DEDFSSIADM DFSALLSQIS
551 S
```

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