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The Evaluation of Cbl as a Therapeutic Target for the Treatment of Cancer

by

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Abstract

Cellular signalling pathways are often misregulated and overactive in cancer cells. Many current cancer therapeutics aim to downregulate these oncogenic signalling pathways. This dissertation summarizes research on the mechanism of Cbl-mediated transformation, the development of Cbl-directed therapeutics, and the applicability of Cbl-directed therapeutics to the treatment of cancer.

The Cbl family of E3 ubiquitin-ligases are known to be involved in cancer; however, the exact nature of their involvement is poorly understood. Cbl protein expression, phosphorylation, and membrane association have each been correlated with poor prognosis in several different cancers. However, our understanding of Cbl's involvement in cellular transformation remains incomplete: the current dominant negative model of Cbl-mediated cellular transformation does not account for all of the existing data and it is currently unclear how wild-type Cbl is involved in cancer. To clarify Cbl's involvement in cellular transformation and human cancer, I propose a new model to explain Cbl-mediated cellular transformation. I evaluate this model by introducing a number of defined mutations into wild-type c-Cbl in order to test the requirements for Cbl-mediated transformation. The characterization of these mutants validates our model and identifies both mutant and wild-type Cbl as potential targets for the treatment of cancer.

While searching for compounds that would therapeutically target the Cbl family of proteins, I demonstrate that piceatannol is able to induce the loss of these proteins. I characterize this, previously unrecognized, property of piceatannol and demonstrate that this phenomenon is not due to the stimulation of known Cbl-regulatory proteolytic pathways. I propose that Cbl loss is mediated directly by piceatannol, through its oxidative conversion into an o-benzoquinone. I validate this hypothesis using an *in vitro* reaction system and demonstrate a novel way in which piceatannol-induced protein loss can be exploited as a cancer therapeutic to inhibit oncogenic signalling pathways.

Collectively, the experiments presented in this dissertation endeavour to clarify the role of Cbl in cellular transformation in order to assess the feasibility of using

piceatannol-like compounds, herein characterized to induce the loss of Cbl, as a therapeutic for the treatment of cancers in which Cbl is prominently involved.

Preface

The work presented in this thesis is currently being prepared for publication. The material from Chapter 3 is being prepared for submission to Oncogene, and the material from Chapters 4 and 5 is being prepared for resubmission to Molecular Cancer Therapeutics.

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To my family.

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Chapter One: General Introduction

1.1 Cancer

Multicellular organisms have an inherent susceptibility to the development of cancer. Multicellularity requires the social cohesion of cells for the greater good of the organism. In cancer, which affects nearly all multicellular organisms, single cells break from their social bonds and proliferate in an uncontrolled and selfish manner, to the detriment of the organism as a whole (Greaves, 2007). The very same evolutionary mechanism that allowed for the progression to multicellularity is therefore the Achilles' heel of these multicellular organisms.

Cancer is the result of the accumulation of somatic mutations that impinge upon the ability of a cell to regulate its birth and death processes (Knudson, 2001). As there are many redundant mechanisms involved in the control of these birth and death processes, mutations in several of these mechanisms are required for carcinogenesis. This is the basis for the multiple hit hypothesis of cancer, which estimates that as few as three and as many as twelve mutations are required to induce neoplastic transformation (Renan, 1993; Knudson, 2001). Exceptions to the multiple hit hypothesis exist; certain cancers have been shown to result from a single mutation, such as the Philadelphia chromosome translocation that generates the BCR-Abl oncogene and causes chronic myelogenous leukemia (CML) (Knudson, 2001; Melo and Barnes, 2007). However, in its early stages, CML is a relatively benign cancer and it is only after the acquisition of additional mutations that this cancer becomes life threatening (Melo and Barnes, 2007); therefore this cancer also lends support to the multiple hit hypothesis. What the multiple hit hypothesis fails to explain is how a cell can accumulate sufficient mutations to become neoplastic, given the low rate of spontaneous somatic mutations in most cells (Knudson, 2001).

1.1.1 Genetic instability and carcinogenesis.

One of the hallmarks of cancer is genetic instability (Balmain, 2001; Knudson, 2001). Thus, one likely explanation for the paradox between the low rate of spontaneous mutation and the relatively high incidence of cancer is that cancer-initiating mutations lead to genetic instability. If these early initiating mutations increased the rate of

acquisition of further mutations, cancers would arise more quickly and frequently (Knudson, 2001). This idea is supported by patients with inherited disorders affecting DNA repair and genetic stability, who are subject to an increased susceptibility to cancer (Eyfjord and Bodvarsdottir, 2005).

Similar to somatic mutations, epigenetic alterations can also facilitate modified gene expression. Epigenetic regulation of gene expression can change the transcription of genes by altering the condensation state of chromatin *via* the post-translational modification of histones or by altering the methylation state of the DNA. Changes in DNA methylation patterns can alter gene expression patterns and the rate of DNA methylation is much more rapid than the rate of somatic mutation (Merlo et al., 2006). Not only has epigenetic silencing of genes been shown in cancer, but hypermethylation of genes involved in genetic stability, such as DNA damage response and repair genes, have been identified in many cancers (Merlo et al., 2006). Therefore epigenetic alterations in cells that neutralize genes associated with genetic stability may be key initiating events in carcinogenesis as they are more likely to occur than somatic mutations and they provide an environment that facilitates the acquisition of further mutations (Merlo et al., 2006). Somatic mutations in genes that control epigenetic stability are thus also likely initiators of carcinogenesis.

1.1.2 Tumour suppressors and oncogenes.

Genomic instability increases the genomic heterogeneity of a tumour population, allowing for an increased rate of tumour evolution and selecting for the most aggressive and resistant clones (Knudson, 2001; Merlo et al., 2006). In addition to genes involved in genetic stability, mutations in two important classes of genes are also known to participate in carcinogenesis and tumour progression: tumour suppressors and oncogenes (Balmain, 2001).

1.1.2.1 Tumour suppressors.

Tumour suppressors are genes that are recessive, requiring the mutation of both alleles to cause phenotypic cellular changes. These genes generally encode proteins involved in regulating cellular checkpoints involved in proliferation, apoptosis and senescence (Michor et al., 2004). Thus, the loss of tumour suppressor gene function

relieves negative regulation of cellular proliferation by circumventing cell cycle regulation and/or apoptosis.

One of the most well known examples of a tumour suppressor gene is the *p53* gene. p53 protein activity is ubiquitously lost in human cancers, either through *p53* gene mutation or through p53 protein inactivation (Bourdon, 2007). In response to cellular stress, such as acute genotoxic stress or oncogene-induced cell cycle progression, p53 induces cell cycle arrest or apoptosis (Bourdon, 2007; Vousden and Lane, 2007). Cell cycle arrest is induced through the p53-dependent upregulation of genes, such as the cell cycle inhibitor *Waf1* (Vousden and Lane, 2007). p53-induced apoptosis occurs through the upregulation of genes involved in the intrinsic apoptotic pathway such as the pro-apoptotic Bcl-2 family protein Bid (Bourdon, 2007; Vousden and Lane, 2007). p53 can also induce apoptosis through its direct association with the anti-apoptotic Bcl-2 family member proteins Bcl-xL and Bcl-2. Bcl-xL and Bcl-2 bind to the pro-apoptotic proteins Bid and Bax, preventing Bid and Bax from heterodimerizing and inducing mitochondrial membrane permeabilization, and thus precipitating apoptosis (Moll et al., 2005). The physical association of p53 with Bcl-xL and Bcl-2 interferes with the ability of these proteins to sequester Bid and Bax resulting in apoptosis (Moll et al., 2005). Therefore, the loss of p53 leads to enhanced cellular proliferation and decreased cell death, strongly promoting tumour growth.

Interestingly, the loss of another tumour suppressor gene, the *Rb* gene, may create a selective pressure for tumour cells to acquire p53 mutations (Symonds et al., 1994). The *Rb* gene encodes a nuclear phosphoprotein that has a demonstrated ability to arrest cells during the G1-phase of the cell cycle by binding to, and repressing, the E2F family of transcription factors (Harbour and Dean, 2000). The E2F family of transcription factors are responsible for the transcription of many proteins involved in the progression of the cell cycle from G1- to S-phase. While the *Rb* gene is only deleted in a small number of cancers, including, retinoblastoma, osteosarcoma, and small-cell lung cancer, it is functionally inactivated via hyperphosphorylation in most other cancers (Harbour and Dean, 2000). Growth factor-mediated mitogenic responses can induce the hyperphosphorylation of Rb, blocking its association with E2F and promoting cell cycle

progression (Harbour and Dean, 2000). This loss of Rb function increases the rate of cell proliferation, often leading to the activation of p53 and the induction of apoptosis (Harbour and Dean, 2000). Accordingly, tumours with wild-type p53 contain many cells undergoing apoptosis; this environment provides a strong selective pressure for the loss of p53 and explains its frequent mutation in cancer (Symonds et al., 1994).

1.1.2.2 Oncogenes.

As mentioned, Rb can be functionally inactivated via hyperphosphorylation (Harbour and Dean, 2000). Rb hyperphosphorylation is primarily mediated by cyclin dependent kinases (CDKs) and the activity of these kinases can be induced by another prominent group of genes associated with carcinogenesis known as oncogenes. Oncogenes are dominant genes that require the mutation of only one allele to cause phenotypic cellular changes. Oncogenes are generally mutated forms of cellular proteins referred to as proto-oncogenes: the oncogenic potential of proto-oncogenes is unleashed *via* their deregulation, amplification or overexpression in normal cells (Stehelin, 1976; Bishop, 1981). Proto-oncogenes have been identified at all levels of cellular signalling cascades involved in the control of cellular growth, proliferation, and differentiation.

At the receptor level, the mutation and/or overexpression of the epidermal growth factor receptor (EGFR) is commonly associated with cancers of epithelial origin; including, breast, colorectal, head and neck, renal cell and non-small cell lung cancer (Bareschino et al., 2007). Elevated and unregulated EGFR signalling is strongly oncogenic as it activates a number of different signalling pathways that redundantly promote cell growth and survival. EGFR-dependent activation of the phosphatidylinositol 3-kinase (PI3K) pathway, the protein kinase C (PKC) pathway, and the Ras/MAPK pathway, in addition to the activation of c-Abl, all strongly promote cellular proliferation (Normanno et al., 2006). EGFR-dependent stimulation of the PI3K pathway, the signal transducers and activators of transcription (STAT) pathway, the Ras/MAPK pathway, as well as the activation of c-Jun N-terminal kinase (Jnk) and c-Abl, strongly promotes cell survival by increasing the level of anti-apoptotic factors and decreasing the function and level of pro-apoptotic factors (Normanno et al., 2006). Interestingly, hyperactivation of the EGFR is also thought to functionally inactivate the

Rb tumour suppressor. This hyperactivation has been shown to release the inhibition of cell cycle progression in a cyclin D1-dependent manner (Kobayashi et al., 2006). This suggests that the EGFR-mediated upregulation of cyclin D1 facilitates the CDK-dependent hyperphosphorylation of Rb, leading to the functional inhibition of this tumour suppressor (Kobayashi et al., 2006).

Bypassing the need for receptors, the BCR-Abl oncogene is an activated fusion mutant of the non-receptor tyrosine kinase (NRTK) signalling effector protein c-Abl. The BCR-Abl oncogene is the causative agent of CML and is also found in approximately 20% of cases of acute lymphocytic leukemia (ALL) (Deininger et al., 2001). It is generated by a translocation between chromosomes 22 and 9, which generates a fusion protein that contains the amino-terminus of the breakpoint cluster region (BCR) protein and the carboxy-terminus of the c-Abl tyrosine kinase (Deininger et al., 2001). Tetramerization of BCR-Abl, mediated by the BCR region, constitutively activates Abl tyrosine kinase activity, leading to the inappropriate tyrosine phosphorylation of a number of Abl associated signalling molecules; including, PI3K, Ras-GAP, and STAT proteins. These tyrosine phosphorylated molecules initiate signalling pathways that promote cell growth and cell survival. In addition, BCR-Abl tyrosine kinase activity is responsible for the functional inhibition of the tumour suppressors, Rb and p53. Inhibition of BCR-Abl by the Abl inhibitor imatinib has been shown to decrease cyclin D1 and cyclin D2 levels and promote cell cycle arrest, suggesting that similar to unregulated EGFR kinase activity, BCR-Abl kinase activity leads to the functional inactivation of Rb in a CDK-dependent manner (Deininger et al., 2001; Fernandez de Mattos et al., 2004). In addition, BCR-Abl is thought to functionally inactivate the p53 tumour suppressor *via* the upregulation of MDM2, a negative regulator of p53 (Levav-Cohen et al., 2005). These observations help to explain the low frequency of Rb and p53 mutation in CML and further demonstrate the importance of BCR-Abl signalling in the initiation and in the malignant progression of CML (Levav-Cohen et al., 2005; Melo and Barnes, 2007).

At the transcriptional level, the c-Myc transcription factor is mutated, overexpressed, or constitutively activated in most cancers (Boxer and Dang, 2001; Evan

et al., 2005). This transcription factor is normally upregulated in response to mitogenic signalling and promotes the transcription of genes involved in cell cycle progression; including *cdc25a*, which encodes a phosphatase involved in CDK activation, as well as *cyclin D2* and *CDK4* (Boxer and Dang, 2001). Activation of c-Myc also leads to the transcriptional repression of other genes such as *p21cip1*, involved in the repression of cell cycle progression (Boxer and Dang, 2001). As a result, the oncogenic activation of c-Myc promotes Rb inactivation and cell cycle progression leading to p53-dependent apoptosis. This presents a strong evolutionary pressure for cancer cells in which c-Myc is constitutively activated to select for mutants with functionally inactive p53 (Eischen et al., 1999; Henriksson et al., 2001).

1.2 Signalling in cancer cells.

In addition to genomic instability, deregulated cellular signalling is also a hallmark of cancer. As has been discussed, many oncogenes are signalling molecules: RTKs, non-receptor tyrosine kinases (NRTKs), serine/threonine kinases or their regulatory subunits, and transcription factors. Evidence suggests that cancers become dependent upon these deregulated and overactivated signalling pathways for their continued survival: inhibitors of many RTKs and NRTKs are currently in use as cancer therapeutics due to their ability to specifically induce apoptosis in cancer cells that are dependent upon signalling pathways driven by those specific protein tyrosine kinases (PTKs) (Baselga, 2006). Perhaps the most successful of these inhibitors is the BCR-Abl kinase inhibitor, imatinib. The introduction of imatinib has revolutionized the treatment of CML, dramatically increasing the average 5-year survival of CML patients from under 70% to over 90% (Sherbenou and Druker, 2007). EGFR specific inhibitors, including monoclonal antibodies and small molecule kinase inhibitors, have also been proven to be successful therapeutic options for cancers that overexpress EGFR (Mendelsohn and Baselga, 2006).

How and why cancers become addicted to these oncogene-induced signalling pathways is not well understood. One reasonable hypothesis proposed by Sharma and colleagues states that as cancers acquire more mutations, pro-apoptotic pathways are induced (Sharma et al., 2006). The activation of these pro-apoptotic pathways provides a strong selective pressure for these cells to acquire mutations that can activate anti-

apoptotic pathways and inactivate pro-apoptotic tumour suppressors. One example of a pathway commonly overactivated in cancer cells, which can accomplish this pro-growth phenotype, is the mitogenic Ras/MAPK pathway (Sharma et al., 2006).

The activation of oncogenic growth factor receptor signalling pathways that can activate anti-apoptotic signalling pathways and inactivate tumour suppressors, can be achieved by various means: autocrine or paracrine stimulation of growth factor receptors, constitutive activation of downstream signal transduction proteins, and growth factor-independent activation of receptors (Klein et al., 2005). Oncogenic mutations are generally responsible for the autocrine activation of growth factor receptors and the constitutive activation of downstream signal transduction proteins. Cancer cell adaptations that promote growth factor-independent activation of receptors include both oncogene-independent mechanisms, such as the increase of reactive oxygen species (ROS) and oncogene-dependent mechanisms, such as the functional loss of negative regulatory proteins.

It has long been appreciated that cancer cells have higher levels of ROS than their normal counterparts (Toyokuni et al., 1995). This is thought to be the result of an evolutionary process whereby cancer cells with increased ROS are selected for because of the numerous, associated, growth and survival advantages that high levels of ROS provide (Finkel, 2003; Loo, 2003). Increased ROS levels in cells can broadly induce RTK signalling activity through the oxidation of active site cysteine residues in protein tyrosine phosphatases (PTPs) (Chiarugi and Cirri, 2003; Loo, 2003; Chiarugi, 2005). The loss of PTP activity, which normally negatively regulates the kinase activity of PTKs, effectively activates growth factor receptor signalling pathways (Chiarugi and Cirri, 2003; Loo, 2003; Chiarugi, 2005).

The loss of negative regulatory proteins involved in growth factor receptor signalling pathways can also lead to the constitutive activation of these pathways. Recently, mutations proximal to the RING finger domain of c-Cbl and Cbl-b, two members of the Cbl family of E3 ubiquitin-ligases, were found in blast cells from AML patients (Caligiuri et al., 2007). The Cbl family of proteins are known to negatively regulate a large number of NRTKs and RTKs by mediating their ubiquitination and

subsequent degradation (Thien and Langdon, 2001; Schmidt and Dikic, 2005). The Cbl deletion mutants found in the AML patients, similar to naturally occurring transforming mutants of Cbl isolated from murine cell lines, are thought to behave in a dominant negative manner by competing with wild-type Cbl (wtCbl) for binding target PTKs, thus preventing their ubiquitination and degradation (Thien and Langdon, 2001). Accordingly, siRNA knockdown of the mutant Cbl proteins decreased the rate of growth of the patient derived AML blasts, demonstrating the involvement of these mutant proteins in cellular proliferation and survival signalling pathways (Caligiuri et al., 2007).

1.3 The c-Cbl proto-oncogene

The c-Cbl proto-oncogene is an adaptor protein and a RING finger E3 ubiquitin-ligase; as such, it acts as both a positive and negative regulator of many receptor and non-receptor tyrosine kinases. Upon activation of a number of different RTKs, c-Cbl is recruited from the cytosol to the activated receptor complex where it recruits signalling effector proteins. Cbl then acts to ubiquitinate these receptors as well as many of their associated signalling proteins (Thien and Langdon, 2001; Schmidt and Dikic, 2005) leading to their degradation by the proteasome and/or lysosome (Thien and Langdon, 2001). Cbl itself is also regulated by ubiquitin-mediated proteolysis (Thien and Langdon, 2001; Yokouchi et al., 2001; Howlett and Robbins, 2002; Magnifico et al., 2003).

In addition to the original identification of the retrovirally encoded v-Cbl oncogene (Langdon et al., 1989), two naturally occurring oncogenic mutant isoforms of c-Cbl have been identified: p95Cbl and 70ZCbl (Andoniou et al., 1994; Bisson et al., 2002). These mutant c-Cbl proteins contain deletions near, or of, the RING finger, which cripple their E3 ubiquitin-ligase activity (Andoniou et al., 1994; Thien and Langdon, 2001; Bisson et al., 2002). As mentioned, these Cbl-mutants are thought to act as dominant negative proteins, competing for substrate signalling molecules with wtCbl and preventing their ubiquitin-mediated downregulation (Swaminathan and Tsygankov, 2006). Interestingly, wtCbl has also been correlated with human cancer: increased Cbl protein levels, increased Cbl tyrosine phosphorylation, and constitutive Cbl membrane association have all been associated with poor prognosis (Brizzi et al., 1998; Ito et al., 2004). This correlation suggests that Cbl's function as a negative regulator may be corrupted by

cancer cells and may indicate that Cbl is solely functioning as a positive regulator of signalling in these cells.

If the function of wtCbl is usurped by cancer cells to promote carcinogenesis, the Cbl family of proteins presents a promising therapeutic target. While Cbl proteins are widely expressed, they are only functionally required in actively signalling cells: Cbl-b and c-Cbl knockout mice have relatively minor phenotypes, largely restricted to B cells and T cells, respectively (Rao et al., 2002; Duan et al., 2004). Therefore, targeting Cbl proteins as a cancer therapeutic would likely have minimal side-effects due to the lack of requirement for Cbl in the normal function of most cells. The association of Cbl family members with many different oncogenic RTKs and NRTKs also makes anti-Cbl therapy broadly specific for many different cancers. There is evidence to support the potential effectiveness of an anti-Cbl therapeutic: the loss of mutant Cbl proteins in patient derived leukemic cells, leads to a reduction in the rate of growth of these cells (Caligiuri et al., 2007). In addition, loss of either c-Cbl or Cbl-b significantly inhibited the migration response of lymphoma cells to the chemokine SDF-1 (Okabe et al., 2006). SDF-1 and its receptor CXCR4 are known to be involved in the metastasis and migration of many different cancers; including, breast cancer, head and neck cancer, small-cell lung cancer, non-small-cell lung cancer, and multiple myeloma (Dorsam and Gutkind, 2007). Targeting Cbl proteins may therefore be an effective therapeutic option, if their role in cancer can be more solidly characterized.

1.4 Cancer therapy

Cancer cells are generated through the mutation or loss of genes that help to regulate the birth and death processes of cells; as such, these cells proliferate more quickly and are able to survive in more adverse conditions than normal cells. Paradoxically, these cells are generally less able to deal with cytotoxic insults than their normal counterparts (Klein et al., 2005). Traditional treatments take advantage of the relative weakness of cancer cells to cytotoxic stressors. Cancers are generally treated with cytotoxic therapies that can kill all cells, but that are more likely to kill cancer cells due to these inherent weaknesses. As these therapies also kill normal cells, side-effects

from normal cell death limit the dosage and thus the overall effectiveness of these treatments (Klein et al., 2005).

Newer therapies target specific molecules thought to be causative or essential for cancer cell growth and survival. These targeted therapies should, in principle, produce fewer side-effects than the traditional cytotoxic therapies. Many of the specific molecules that are targeted by these therapies are kinases, which are persistently active and required for the proliferation and survival of cancer cells but not for normal cells (Baselga, 2006). Some of these targeted therapies are now clinically approved for the treatment of specific cancers (Chabner and Roberts, 2005; Klein et al., 2005). For example, the BCR-Abl inhibitor imatinib is currently used for the treamtment of CML (Sherbenou and Druker, 2007), and the EGFR inhibitor erlotinib as well as the VEGFR directed monoclonal antibody bevacuzimab have both been approved for the treatment of non-small-cell lung cancer (Bareschino et al., 2007; Sandler, 2007). Results from clinical trials using these targeted therapies have identified two major shortcomings. Firstly, these therapeutics are not effective in the treatment of late stage cancers: these tumours have acquired a significant number of mutations and therefore sub-populations of these tumours are no longer dependent upon the targeted signalling pathways for survival (Chabner and Roberts, 2005; Arbiser, 2007). Secondly, by specifically targeting the kinase domains of causative oncogenes, these therapeutics select for cancer cells with mutations in the kinase domains that are resistant to inhibition (Chabner and Roberts, 2005; Arbiser, 2007). For example, the causative mutation in CML leads to the production of a single oncogene, BCR-Abl, which is sufficient to induce cellular transformation. Targeting the activity of this mutant kinase with the specific inhibitor imatinib can lead to remission of the cancer, but ultimately imatinib-resistant cancer clones overcome the therapy (Roche-Lestienne and Preudhomme, 2003; Klein et al., 2005).

What has been determined from many of the single inhibitor studies is that combination therapies are the best approach, as they take advantage of specific oncogenic pathways while also limiting the selection of cancer cells that are resistant to therapy (Klein et al., 2005; Araujo et al., 2007; Arbiser, 2007). Another therapeutic option

combines the use of newer targeted therapies with older cytotoxic regimens. This approach takes advantage of the ability to inhibit specific, essential survival factors of a given cancer, thus sensitizing the cancer to lower doses of chemotherapy, and reducing the side effects of treatment (Klein et al., 2005; Bareschino et al., 2007).

Newer therapeutics are also being developed with broader specificity in mind. These therapeutics are still targeted to cellular functions that promote cancer cell specificity. However, their targets are key molecules involved in the regulation of numerous oncogenic signalling pathways. For example, inhibitors that block the function of HSP90 affect the function of numerous HSP90 client proteins including EGFR, CDK4, and BCR-Abl (Bagatell and Whitesell, 2004). As well, broad spectrum kinase inhibitors target multiple oncogenic kinases simultaneously are under development: AMG 706 has been shown to be an ATP-competitive inhibitor of the kinase activity of vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor, and stem-cell factor receptor (Rosen et al., 2007).

It is also possible to target cancer by taking advantage of the persistent state of oxidative stress commonly associated with cancer (Toyokuni et al., 1995). As all cells have only a limited capacity to deal with ROS, increasing the amount of ROS in cancer cells allows ROS-inducing therapeutics to induce cell cycle arrest and apoptosis (Loo, 2003). One major advantage to ROS-inducing therapeutics is that they generally have little effect on the surrounding normal tissue, because the normal cells present in this tissue are able to easily buffer the increase in free radicals (Loo, 2003). ROS production by thalidomide, arsenic trioxide and proteasome inhibitors such as bortezomib have all been shown to be required for their efficacy as cancer therapeutics (Hideshima and Anderson, 2002; Ling et al., 2003; Amadori et al., 2005; Engel and Evens, 2006; Ge et al., 2006; Baysan et al., 2007).

While many of the current therapeutic options are able to prolong the lives of cancer patients, cancer is a constantly evolving disease that requires constantly evolving therapies to manage it. As such, there is a continuing need for new and innovative strategies to treat cancer.

1.5 Objectives

This dissertation summarizes research on the mechanism of Cbl-mediated transformation, the development of Cbl-directed therapeutics, and the applicability of Cbl-directed therapeutics to the treatment of cancer. The first part of this dissertation, presented in Chapter 3, examines the mechanism of Cbl-mediated transformation in order to determine the feasibility of targeting Cbl as a therapeutic option for the treatment of cancer. The Cbl family of proteins are known to be involved in cancer; however, the exact nature of their involvement is poorly understood. Cbl protein expression, phosphorylation, and membrane association have been correlated with poor prognosis in several different types of cancer. In addition, Cbl mutants found in murine cell lines have been shown to induce cellular transformation and similar mutants have recently been identified in AML cells isolated from humans. However, our understanding of Cbl's involvement in cellular transformation remains incomplete: the current dominant negative model of Cbl-mediated cellular transformation does not account for all of the existing data and it is currently unclear how wild-type Cbl is involved in cancer. To clarify Cbl's involvement in cellular transformation and human cancer, I propose a new model to explain Cbl-mediated cellular transformation. I evaluate this model by introducing a number of defined mutations into wild-type c-Cbl in order to test the requirements for Cbl-mediated transformation. The characterization of these mutants validates our model and identifies both mutant and wild-type Cbl as potential targets for the treatment of cancer.

The second part of this dissertation, presented in Chapter 4, seeks to identify compounds that target Cbl proteins and to determine the mechanism of action of these compounds. In this chapter I demonstrate that the hydroxystilbene, piceatannol, and related catechol-ring containing compounds, are able to induce the loss of the Cbl-family of proteins. I characterize this previously unrecognized property of piceatannol to determine the feasibility of using piceatannol-like compounds as cancer therapeutics. After ruling out a mechanism of piceatannol-induced Cbl loss based on the known Cbl-regulatory proteolytic pathways, I propose that this Cbl loss is mediated directly by piceatannol through its oxidative conversion into a highly reactive o-benzoquinone. This

hypothesis is validated using a well defined *in vitro* reaction system using the minimum requirements for piceatannol-induced Cbl loss. I further characterize the protein selectivity of piceatannol-induced protein loss and demonstrate the functional loss of Cbl-associated protein involved in signalling pathways commonly deregulated in cancer. These experiments establish the utility and applicability of using piceatannol-like compounds for the treatment of cancers involving Cbl.

The final part of this dissertation, presented in Chapter 5, tests the feasibility of using compounds that induce the loss of Cbl as cancer therapeutics. Piceatannol's usefulness as a cancer therapeutic is tested in multiple myeloma cell lines. Multiple myeloma cells were selected as a model cancer to test piceatannol's therapeutic potential due to their sensitivity to piceatannol-induced cell death, due to their dependence on Cbl-dependent signalling processes for disease associated morbidity and chemo-resistance, and due to the potential synergy between piceatannol and existing multiple myeloma therapeutics. I evaluate the feasibility of this treatment for multiple myeloma by using an *in vitro* model system. I demonstrate piceatannol's selective killing and inhibition of Cbl-dependent migration of cancer cells.

Collectively, the experiments presented in this dissertation endeavour to clarify the role of Cbl in cellular transformation in order to assess the feasibility of using piceatannol-like compounds, herein characterized to induce the loss of Cbl, as a therapeutic for the treatment of cancers in which Cbl is prominently involved.

Chapter Two: Materials and Methods

2.1 Solutions

Agarose Gel Electrophoresis

TAE: 40mM Tris acetate, 1mM ethylenedinitrilo tetraacetic acid (EDTA), pH 8.0

6x DNA loading buffer: 30% (v/v) glycerol, 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 100mM EDTA pH 8.0

Gelatin Zymography

Rinse buffer: 50mM Tris pH7.5, 5mM $CaCl_2$, 3.86% (v/v) Triton X-100

Incubation buffer: 50mM Tris pH7.5, 5mM $CaCl_2$

Coomassie stain: 30% (v/v) isopropanol, 10% (v/v) acetic acid, 2.5mg/mL Coomassie blue

Destain: 10% (v/v) isopropanol, 10% (v/v) acetic acid

4x Zymography loading buffer: 40% (v/v) glycerol, 4% (w/v) sodium dodceyl sulphate (SDS), 200mM Tris base pH 6.8, bromophenol blue to colour

Immunoprecipitations

SDS/DOC wash buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 0.1% SDS, 0.1% deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 µg/mL of each aprotinin and leupeptin

Phosphate-buffered saline (PBS): 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 154 mM NaCl, pH 7.4

In Vitro Kinase Assay

Kinase assay buffer: 50 mM Tris base pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 µM ATP

Lipid Raft Isolation

Mes-buffered saline (MBS): 25mM morpholineethanesulfonic acid (Mes) pH 6.5, 150mM NaCl

Triton-MBS lysis buffer: 1% Triton X-100 in MBS, 1mM sodium orthovanadate, 10 µg/mL each of aprotinin and leupeptin, 1mM PMSF

Lysis Buffers

Hypotonic lysis buffer: 10 mM Tris base pH 8.0, 10 mM KCl, 1 mM EDTA; 0.1 mM β-mercaptoethanol, 0.01 mg/mL each of aprotinin and leupeptin, 1 mM PMSF and 1mM sodium orthovanadate were added immediately before use.

NP40 lysis buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Nonidet P-40 (protein grade), 1 mM PMSF, 1 mM sodium orthovanadate, and 10 μg/mL each of aprotinin and leupeptin

2x Laemmli sample buffer: 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 125 mM Tris base pH 6.8, 0.002% bromophenol blue

SDS-PAGE

Acrylamide: 29.2% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide in double-distilled (dd)H_2O, filtered

Gel running buffer: 25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS

4x Lower gel buffer: 1.5 M Tris, 0.4% (w/v) SDS, pH 8.8

4x Upper gel buffer: 0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS

4x Laemmli sample buffer: 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 8% (w/v) SDS, 250 mM Tris base pH 6.8, 0.004% bromophenol blue

Transfer buffer: 25 mM Tris base, 192 mM glycine, 20% methanol, 0.1% SDS

Western Blotting

Tris-buffered saline (TBS): 5mM Tris base, 135 mM NaCl, 5 mM KCl

TBS-Tween: 1x TBS, 0.1% (v/v) Tween-20

ECL: Solution 1: 30% (v/v) H_20_2, 10 mM Tris, pH 8.5; Solution 2: 2.5 mM luminol, 0.4 mM coumaric acid

Stripping buffer: 0.05% (w/v) sodium azide, TBS-Tween

2.2 Chemical reagents

2.2.1 Chemicals

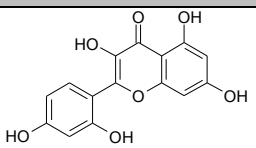
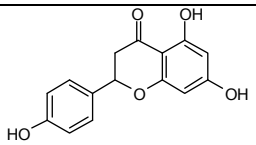
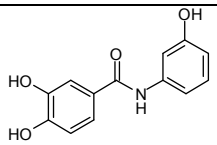
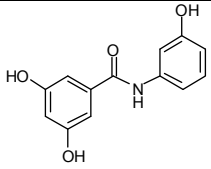
Unless otherwise indicated for specific experiments, cells were incubated for the following times at the following concentrations of chemical reagent prior to cell stimulation, cell treatment, or cell lysis. Also indicated are the general function and the supplier of each chemical.

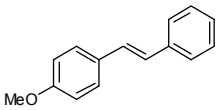
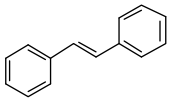
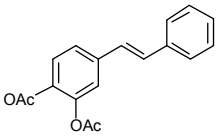
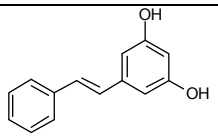
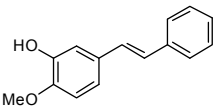
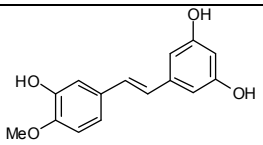
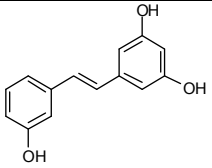
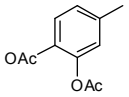
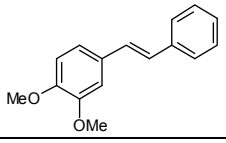
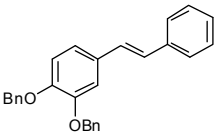
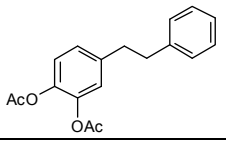
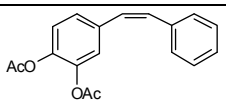
Name	Function	Concentration	Time	Supplier
AG1296	PDGFR inhibitor	10μM	3 hours	Calbiochem
AG1478	EGFR inhibitor	10μM	3 hours	Calbiochem
ALLN	Proteasome inhibitor	10μM	4 hour	Calbiochem
Ammonium chloride	Lysosomal inhibitor	20mM	4 hour	BDH
Bafilomycin A1	Lysosomal inhibitor	200nM	4 hour	Calbiochem
Bisindolylmaleimide I	PKC inhibitor	2μM	3 hours	Calbiochem
Boc-D-FMK	Broad spectrum caspase inhibitor	50-100μM	2 hour	Calbiochem
Curcumin	Antioxidant	50μM	3 hours	Calbiochem
Epigallocatechin gallate	Antioxidant	200μM	3 hours	BioMol
Epoxomycin	Proteasome inhibitor	200nM	4 hour	Calbiochem
EST	Lysosomal inhibitor	25μM	4 hour	Calbiochem
Geldanamycin	Broad spectrum kinase inhibitor	2.5μM	3 hours	Alamone Labs
Genistein	Broad spectrum kinase inhibitor	50μM	3 hours	Calbiochem
Herbimycin A	Broad spectrum kinase inhibitor	1μM	3 hours	GIBCO
LY294002	PI3K inhibitor	10μM	3 hours	Calbiochem
MG132	Proteasome inhibitor	25μM	4 hour	Calbiochem
N-acetyl cysteine	Antioxidant	5mM	3 hours	Sigma
PD98059	MEK inhibitor	25μM	3 hours	Calbiochem
Piceatannol	Kinase inhibitor, antioxidant, apoptosis	2-200μM	as indicated	Sigma, Biomol
PP1	Src-family kinase inhibitor	10μM	3 hours	Calbiochem
PP2	Src-family kinase inhibitor	10μM	3 hours	Calbiochem
Resveratrol	antioxidant, apoptosis	200μM	3 hours	Sigma, Biomol
SB203580	p38MAPK inhibitor	10μM	3 hours	Calbiochem

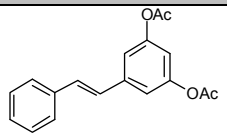
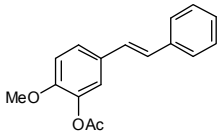
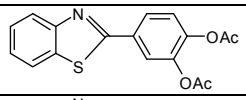
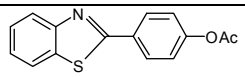
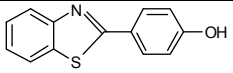
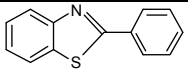
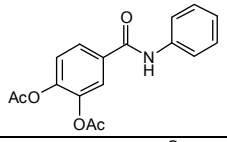
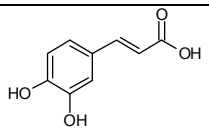
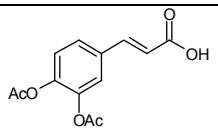
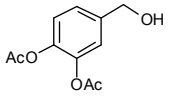
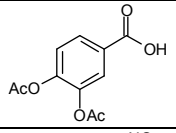
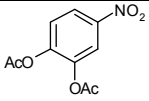
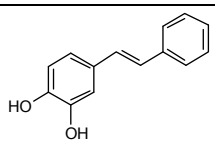
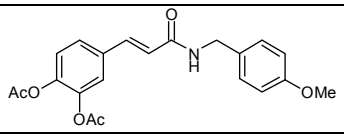
Name	Function	Concentration	Time	Supplier
STI571	Abl kinase inhibitor	1μM	8 hours	Dr. Aru Narendran
SU6656	Src-family kinase inhibitor	10μM	3 hours	Calbiochem
U0126	MEK inhibitor	10μM	3 hours	Calbiochem

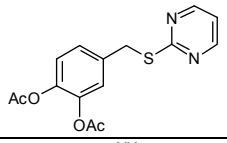
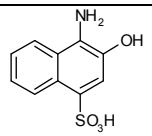
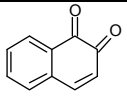
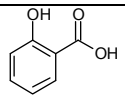
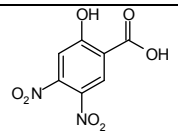
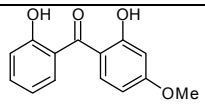
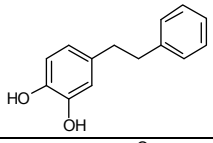
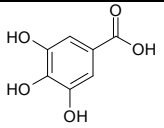
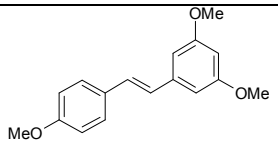
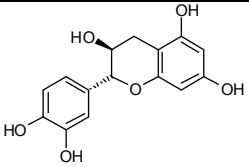
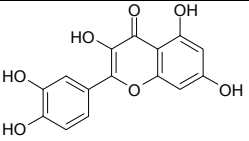
2.2.2 Synthetic piceatannol-like compounds

All compounds were solubilised in DMSO at a stock concentration of 200mM and were stored as individual aliquots at -20°C until use. The following chart details all of the compounds and provides their commonly used reference number, as well as their official names and chemical structures. Compound names beginning with HCH were synthesized by Dr. Henrik Hansen at the University of Calgary. Compound names beginning with KH were synthesized by Karm Hansk at the University of Calgary. Compound names beginning with GL were synthesized by Gavin Leonard at the University of Calgary. Compound 38 was synthesized by Gennady Shustov at NAEJA in Edmonton, Alberta. Compounds without prefixes were purchased from Sigma unless otherwise indicated.

Reference Number	Compound Name	Structure
1	HCH109A (Morin)	
2	HCH109B (Naringenin)	
3	HCH95A	
4	HCH95B	

Reference Number	Compound Name	Structure
5	KH427	
6	Trans-stilbene (Henrik Hansen)	
7	KH573B, KH573C, KH679	
8	KH551	
9	KH565	
10	KH595	
11	KH609	
12	KH759	
13	KH667	
14	KH669	
15	KH681	
16	KH771	

Reference Number	Compound Name	Structure
17	KH777	
18	KH779	
19	GL045	
20	GL059	
21	GL053	
22	GL027	
23	GL061	
24	HCH193	
25	HCH191B	
26	HCH189	
27	HCH179B	
28	HCH183B	
29	HCH197	
30	HCH195A	

Reference Number	Compound Name	Structure
31	HCH241A	
32	HCH257A	
33	HCH257B	
34	HCH257C	
35	HCH259A	
36	HCH259B	
37	HCH259C	
38	6086-2	
Gal	Gallic acid (Henrik Hansen)	
TMR	Trismethoxy resveratrol	
Cat	(+)-Catechin	
Que	Quercetin	

2.3 Molecular constructs

2.3.1 Generation of membrane targeted c-Cbl constructs

A wild type c-Cbl retroviral expression plasmid in the pBabepuro3 vector (Howlett and Robbins, 2002) was used as a template to add an in frame amino-terminal XhoI site, which replaces Cbl's start codon (primer XhoCbl: 5'-GTGGACTCGAGG CCGGCAACGTGAAG-3'), and a carboxy-terminal ClaI site (primer CblCla: 5'-CCATCGATCTAGGTAGCTACATGG-3'). This PCR product was digested with XhoI and ClaI and ligated into pBabepuro3 to generate pBabe/Cbl^{XC}. The PCR product was also used in a second PCR reaction to splice it together with another PCR product containing a Kozak sequence and 153 base pairs from the 5'-coding region of rat Csk binding protein (Cbp) from the pK1-Cbp plasmid (primer rCBP(N)s: 5'-GTAGGATCCGCCGCCACCATGGGACCTGCAGGA-3' and primer rCBP(N)a: 5'-CGGCCTCGAGTCCACTATGCTGCCG-3'). The two PCR products were spliced together using the primers rCBP(N)s and CblCla, and the resultant PCR product was digested with BamHI and ClaI and ligated into pBabepuro3 to generate pBabeCbpCbl. The C381A mutation was introduced into pBabe/Cbl^{XC} and pBabeCbpCbl by replacing the 0.7kb BlpI-BglII fragment from these constructs with the corresponding fragment from the pSX/Cbl^{C381A}, which was kindly donated by Drs. C. Joazeiro and T. Hunter (The Salk Institute, La Jolla, CA). Cassette ligation primer pairs containing a Kozak sequence followed by 48 base pairs from the 5'-coding region of mouse c-Src (primer Src(N)s: 5'-GATCCGCCGCCACCATGGGTAGCAACAAGAGCAAGCCCAAGGATGCCAGCC AGCGGCGCCGCC-3', and primer Src(N)a: 5'-TCGAGGCGGCGCCGC TGGCTGGC ATCCTTGGGCTTGCTCTTGTTGCTACCCATGGTGGCGGCG-3') were then ligated into BamHI/ XhoI digested pBabe/Cbl^{XC} and pBabe/Cbl$^{XC\text{-}C381A}$ generating pBabe/SrcCbl and pBabe/SrcCbl^{C381A}. To add a Kozak sequence, and to properly control for the XhoI site introduced immediately 5' of the second amino acid of Cbl in the CbpCbl and SrcCbl constructs, a cassette ligation primer pair to introduce an identical XhoI site in-between c-Cbl's start codon and its second codon (primer WtCbl(N)s: 5'-GATCCGCCGCCAC CATGC-3', and primer WtCbl(N)a: 5'-TCGAGCATGGTGGCGG CG-3') was ligated into BamHI/XhoI digested pBabe/Cbl^{XC} generating pBabe/WtCbl(N). pBabe/Cbl$^{\Delta 1\text{-}355}$, a

c-Cbl amino-terminal truncation mutant that begins at codon 356, was generated by PCR using pBabe/WtCbl(N) and an amino-terminal primer introducing a XhoI site, in front of c-Cbl codon 356 (primer XhoCbl356(s): 5'-CCTCGAGACTCCCCAAGACCATATC-3', and primer Cbl6(a): 5'-TCTCTGGAGGGACAGTCGC-3'). This PCR product was digested with XhoI and BglII, and ligated into XhoI/BglII digested pBabe/WtCbl(N). 70ZCbl and v-Cbl cDNAs, kindly provided by Dr. W.Y. Langdon (University of Western Australia, Nedlands, Australia), were subcloned into the BamHI site in the pBabepuro3 vector. pBabepuro3/p95Cbl was generated as previously described (Bisson et al., 2002).

2.3.2 Site directed mutagenesis

Point mutations were introduced using DpnI-mediated site-directed mutagenesis. Template pBabe plasmids were used in PCR reactions with Pfu DNA polymerase and complementary primers with a melting point of 78°C, containing 12-15 base pairs on either side of the point mutaitons. 1µL of DpnI was added to each PCR reaction and they were incubated for 1 hour at 37°C. DpnI treated DNA was then transformed into TOP10F' competent bacteria and colonies were screened for the inserted point mutations by DNA sequencing.

Mutation	Template Plasmids	Primer Pair
Cbl G306E	pBabe/WtCbl(N) pBabe/70ZCbl pBabe/SrcCbl pBabe/ScrCbl^{C381A}	G306E-s: 5'-TGGGCTATTGAGTACGT AACTGCTGAT-3' G306E-a: 5'-ATCAGCAGTTACGTACT CAATAGCCCA-3'
Cbl Y368F/Y371F	pBabe/WtCbl(N) pBabe/CblC381A	YYFF(s): 5'- CAGGAACAATTTGAAT TATTCTGTGAGATG-3' YYFF(a): 5'-CATCTCACAGAATAAT TCAAATTGTTCCTG-3'
Cbl Y368D/Y371D	pBabe/WtCbl(N) pBabe/CblC381A	YYDD-s: 5'-CAGGAACAAGATGAAT TAGACTGTGAGATG-3' YYDD-a2: 5'-CATCTCACAGTCTAAT TCATCTTGTTCCTG-3'

2.4 Cell culture

2.4.1 Maintenance of cell lines

The following table indicates the growth media and supplements used in the maintenance of all of the cell lines used. Cells that were cultured in DMEM were grown in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin sulphate (GIBCO). Murine 3T3 fibroblasts stably expressing Cbl proteins were cultured in supplemented DMEM with 2µg/mL puromycin. Cells that were cultured in RPMI complete media were grown in RPMI 1640 media (GIBCO) supplemented with 1mM sodium pyruvate (GIBCO), 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO), 50µM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine serum (GIBCO). All cells were maintained in a humidified incubator at 37°C with 5% CO_2. Two independent clones of each 3T3 cell line were used for the experiments to ensure reproducible results. The Cbl-/- mouse embryonic fibroblasts (MEFs) and the wild type Cbl+/+ control MEFs were kindly provided by Dr. Hamid Band (Andoniou et al., 2000). The normal human bone marrow stromal cells (NHBSCs) were kindly provided by Dr. Aru Narendran and were maintained in Opti-MEM I reduced serum media (GIBCO) supplemented with 20% (v/v) fetal bovine serum, 100U/mL penicillin and 100 µg/mL streptomycin.

Cell line name	Species	Origin	Growth Media
3T3	Mouse	Fibroblast	DMEM
70Z/3	Mouse	Pre-B cell lymphoma	RPMI complete
8226	Human	Multiple myeloma	RPMI complete
A431	Human	Squamous cell carcinoma	DMEM
c-Cbl$^{-/-}$ MEF	Mouse	Mouse embryonic fibroblast	DMEM
c-Cbl$^{+/+}$ MEF	Mouse	Mouse embryonic fibroblast	DMEM
HCT116	Human	Colon carcinoma	DMEM
HeLa	Human	Cervical carcinoma	DMEM
HT1080	Human	Fibrosarcoma	DMEM
HTB 126	Human	Mammary gland ductal carcinoma	DMEM

Cell line name	Species	Origin	Growth Media
HTB 129	Human	Mammary gland ductal carcinoma	DMEM
HTB 132	Human	Mammary gland adenocarcinoma	DMEM
J774A.1	Mouse	Reticulum cell sarcoma	RPMI complete
Jurkat	Human	Acute T cell leukemia	RPMI complete
K562	Human	Erythroleukemia	RPMI complete
LAN-1	Human	Neuroblastoma	DMEM
MDA-MB-231	Human	Mammary gland epithelial cell adenocarcinoma	DMEM
MM1.S	Human	Multiple myeloma	RPMI complete
NHBSC	Human	Primary normal human bone marrow stromal cells	OptiMem
OPM2	Human	Multiple myeloma	RPMI complete
Raji	Human	Burkitt's lymphoma	RPMI complete
Ramos	Human	Burkitt's lymphoma	RPMI complete
U251N	Human	Glioblastoma	DMEM
U266	Human	Multiple myeloma	RPMI complete
U87	Human	Glioblastoma	DMEM

2.4.2 Retrovirus production and generation of stably infected cell lines

The Bosc23 murine leukemia virus packaging cell line was transfected with pBabepuro3 retroviral expression constructs using Fugene 6 (Roche), as per the manufacturer's instructions. 24 hours after transfection, the cells were changed into fresh DMEM/ 10% FBS/ 100 U/mL penicillin and 100 µg/mL streptomycin. After 48 hours, the virus containing media was filtered through a 0.2µM syringe filter, aliquoted, and stored at -80°C until use.

Murine 3T3 fibroblasts were infected with retrovirus as they were being passaged. 3T3 fibroblasts were trypsinized, counted using a haemocytometer, and plated at a

density of $5x10^4$ cells/ well in a 6 well dish in 1mL of DMEM/ 10% FBS/ 100 U/mL penicillin and 100 µg/mL streptomycin. 1mL of virus supernatant with 8µg/mL polybrene was added to each well, and the plates were placed in a tissue culture incubator. After 24 hours, the virus containing media was replaced with 2mL of fresh media. After a further 24 hours, the cells were trypsinized and replated into three 15cm tissue culture dishes at increasing cell densities in media supplemented with 2µg/mL puromycin (Sigma). 6 to 12 colonies were picked for each viral construct and grown in duplicate wells in a 24 well dish until confluent. Clones were then screened by Western blotting for Cbl expression and three high expressing clones were selected, passaged, and later stored at -80°C.

2.4.3 Soft Agar Colony Forming Assay

To assess anchorage-independent growth, $5x10^3$ murine 3T3 fibroblast cells/well in were seeded in phenol-red-free DMEM containing 10% charcoal stripped FBS and 0.35% low-melting temperature (LMP) agarose in 6-well dishes. This suspension was sandwiched between layers of DMEM containing 0.7% LMP agarose. Once visible colonies were formed in the positive control wells (after 2-3 weeks), 1mL of PBS with 0.1mg/mL thiazolyl blue tetrazolium bromide (MTT) was added to each well and the dishes were replaced in the tissue culture incubator overnight. The next day, the wells were suctioned dry and documented with the BioRad Gel Doc 2000 using the Quantity One 4.0.3 software. Images of each well were then processed using the colony counting function of Quantity One using the following parameters: Averaging 5, Sensitivity 7.2. Colony counts were adjusted by hand to include missed colonies and to exclude non-colonies. Results are expressed as number of colonies formed per well. Data was averaged from 3 independent experiments performed in triplicate and was expressed graphically as mean ± SEM. A one-way ANOVA and a Student's t-test with Bonferroni correction were used for multiple comparisons.

2.4.4 LC_{50} determination

Adherent cells were plated in a 96 well dish at a density of $5x10^4$ cells per well. The following day, the media on the cells was replaced with fresh media containing the indicated doses of specific piceatannol-like compounds, or DMSO as a control. After a

48 hour or 72 hour incubation, cells were rinsed in PBS and incubated for 1 hour in 50µL of PBS containing 1mg/mL MTT. Cells were lysed overnight at 37°C with the addition of 50µL of 20% SDS/ 50% dimethylformamide (DMF). Absorbance at 595nm was read and values were normalized to the DMSO control. LC_{50} was determined mathematically using the formula: $C_1+((50-X_1)*(C_2-C_1)/(X_2-X_1))$, where C_1 indicates the experimentally tested concentration closest to the LC_{50} that was unable to kill 50% of the cells, where C_2 indicates the experimentally tested concentration closest to the LC_{50} that was able to kill more than 50% of the cells, and, where X_1 and X_2 are the percentage of cells that survived treatment with the concentrations of compound C_1 and C_2, respectively.

Non-adherent cells were plated in 100µL of RPMI complete in round bottom 96 well dishes at a density of $5x10^4$ cells per well. Wells were then treated with 100µL of RPMI complete containing 2x concentrations of the indicated doses of piceatannol-like compounds, or DMSO as a control. After 48 hours the number of viable cells in each well was counted by trypan blue exclusion. Cell numbers were normalized to DMSO controls and LC_{50} values were determined mathematically as above.

2.4.5 Migration Assay

Jurkat and OPM2 cells were diluted to a concentration of $2.5x10^5$ cells/mL in RPMI complete. 2mL of cells were aliquoted for each condition. Aliquots of cells were incubated with the indicated concentrations of piceatannol or DMSO for 1 or 3 hours. 25µL of control RPMI complete or 25µL of RPMI complete containing 100ng/mL of synthetic CXCL12 was added to the lower chamber of 48-well mircochemotaxis chambers (Neuroprobe). The lower chamber was separated from the upper chamber by a polycarbonate membrane with a 5µM pore size (Neuroprobe). 50µL of pretreated cells (12500 cells) were added to the upper chamber, and the apparatus was incubated in a tissue culture incubator for 3 hours. The contents of the upper wells were aspirated and 10µL aliquots from the lower chamber were mixed with an equal volume of 0.4% trypan blue solution (Invitrogen) and the total number of live cells was counted. The total number of cells migrated per well was determined by multiplying the number of cells counted by 2 to account for the dilution with trypan blue and by 2.5 to account for the total volume of the lower chamber. The migration numbers were normalized to the

number of cells migrated in the presence of CXCL12 and the means were plotted with SEM. A one-way ANOVA and a Student's t-test with Bonferroni correction were used for multiple comparisons.

2.5 Cell stimulations and preparation of lysates

Murine 3T3 fibroblasts ectopically expressing Cbl, A431 human squamous cell carcinoma cells, and J-774A.1 murine reticulum cell sarcoma cells were plated in 6-well plates with $5x10^5$ cells per well in 2mL of media, respectively, and grown overnight prior to their use in experiments. Cbl MEFs were plated in 6-well plates with $1x10^5$ cells per well in 2mL of media. K562 and 70Z/3 cells where resuspended at $8x10^5$ cells per mL in media in a total volume of 4 mL in 5mL round bottom polystyrene tubes the day the were used in experiments. Specific details regarding each experiment are included in the figure legends. At the indicated times, cells were rinsed once with PBS and lysed in NP40 lysis buffer. Insoluble material was removed by centrifugation at 16000x g for 5 min at 4°C. Prior to analysis, total protein in the cell lysates was measured using a colorimetric BCA protein assay (Pierce) against bovine serum albumin standards. Alternatively cells were washed in PBS and lysed in hot 2x Laemmli's sample buffer.

2.5.1 Membrane Fractionation

$1x10^6$ murine 3T3 fibroblast cells were plated overnight in a 10cm dish. The following day, cells were washed once in cold hypotonic lysis buffer and then lysed for 20 minutes at 4°C in 1mL of cold hypotonic lysis buffer with freshly added inhibitors and β-mercaptoethanol. Cell lysates were scraped from the dish and Dounce homogenized for 75 strokes on ice. Lysates were then adjusted to a final concentration of 0.25M sucrose and 1mM EDTA. Nuclei and unlysed cells were pelleted by centrifugation at 1000x g for 10 minutes at 4°C. Cellular membranes were pelleted from the post nuclear supernatants by centrifugation at 100,000x g for 1 hour at 4°C. The supernatant was removed and labelled as the cytosolic fraction. The pellet, labelled as the membrane fraction, was washed once in hypotonic lysis buffer with inhibitors and lysed in 100µL of 2x Laemmli buffer. The cytosolic fraction was TCA precipitated through the addition of 1/10th volume of 100% trichloroacetic acid. After a 30 minute incubation on ice, the TCA precipitation was centrifuged at 16000x g for 30 minutes at 4°C. The pellets were

washed 3 times in PBS and lysed in 100µL of 2x Laemmli buffer. Membrane and cytosolic lysates were sonicated for 10 seconds to solubilise the pellets and then heated to 80°C for 6 minutes to denature proteins. 10µL of each membrane and cytosolic fraction was loaded on a 10% SDS-PAGE gel for Western blotting.

2.5.2 Isolation of lipid rafts

Triton X-100-insoluble complexes were isolated as previously described (Robbins et al., 1995). $1x10^8$ 70Z/3 cells or U937 cells were lysed in 2mL of cold Triton-MBS lysis buffer. Lysates were Dounce homogenized for 50 strokes on ice and mixed into an equal volume of 80% sucrose in Triton-MBS lysis buffer. Samples were transferred to Ultra-Clear ultracentrifuge tubes (Beckman), overlayed with a 5-30% linear sucrose gradient, and ultracentrifuged in an SW41 rotor (Beckman) at 55000x g for 16 hours at 4°C. The buoyant fraction, containing lipid rafts, was collected by harvesting the top 7mL of the gradient. The buoyant fractions were diluted 1:3 in MBS, and lipid rafts were pelleted by ultracentrifugation in an SW41 rotor at 55000x g for 1 hour at 4°C. Lipid raft pellets were resuspended in cold kinase assay buffer and stored at -20°C until use.

2.6 Antibodies

The antibodies used for Western blotting and immunoprecipitations are listed in the table below with their specificity and source, the dilutions used, the specific applications they were used for, and their individual sources.

1° Antibody	Dilution	Application	Source
α-c-Abl, rabbit pAb	1:1000	WB	Cell Signaling
α-Actin, mouse mAb	1:5000	WB	Chemicon
α-Cbl-b, mouse mAb	1:1000, 1:100	WB, IP	Santa Cruz
α-c-Cbl, mouse mAb (7G10)	1:2000, 1:200	WB, IP	C.Howlett & S.Robbins, U of Calgary
α-c-Cbl, rabbit pAb	1:1000	WB	Santa Cruz
α-EGFR, mouse mAb	1:1000	WB	Transduction Labs
α-Flotillin-2, mouse mAb	1:1000	WB	Transduction Labs
α-Grb2, mosue mAb	1:1000	WB	Transduction Labs

α-HA, mouse mAb (12CA5)	1:1000, 1:100	WB, IP	S.Robbins, UofC
1° Antibody	Dilution	Application	Source
α-HA, rat mAb (3F10)	1:2000	WB	Roche
α-p56Lyn, rabbit pAb	1:1000, 1:100	WB, IP	A.L.Defranco, UCSF
α-PARP, mouse mAb (C2-10)	1:1000	WB	Trevigen
α-PDGFRβ, rabbit pAb	1:1000, 1:100	WB, IP	Santa Cruz
α-Phospho-tyrosine, mouse mAb (4G10)	1:1000	WB	S.Robbins, U of Calgary
α-Pyruvate Kinase, goat pAb	1:5000	WB	Chemicon
α-Src, mouse mAb (Src327)	1:1000, 1:100	WB, IP	J.M.Bishop, UCSF
α-Src, rabbit pAb	1:1000	WB	Santa Cruz
α-Syk, mouse mAb	1:1000	WB	Santa Cruz
α-Syk, rabbit pAb	1:1000	WB	Santa Cruz
α-Ubiquitin, mouse mAb	1:1000	WB	Zymed
2° Antibody	Dilution	Application	Source
Donkey α-Goat IgG-HRP	1:10000	WB	Santa Cruz
Goat α-Mouse IgG-HRP	1:10000	WB	Santa Cruz/ BD Pharmingen
Goat α-Rabbit IgG-HRP	1:10000	WB	Santa Cruz
Mouse α-Rabbit light chain IgG-HRP	1:10000	WB	Jackson Immunoresearch Labs
Goat α-Rat IgG-HRP	1:10000	WB	Santa Cruz

2.7 Immunoprecipitations

NP-40 cell lysates were Dounce homogenized 50 strokes on ice and clarified by centrifugation at 16000x g at 4°C for 3 minutes. Clarified lysates were pre-cleared by a 45 minute incubation at 4°C with protein G-sepharose (Sigma) and a 1:200 dilution of

normal mouse IgG or pre-immune rabbit serum, depending on the source of the immunoprecipitating antibody. Pre-cleared lysates were then incubated for 1 hour with the α-Cbl 7G10 mAb, or overnight with the α-Cbl-b pAb, the α-p56Lyn pAb, the α-Src327 mAb, the α-HA 12CA5 mAb or the α-PDGFRβ pAb. Protein G-sepharose was then added to the immunoprecipitations and they were incubated for an additional hour at 4°C. The immunoprecipitations were then washed twice in NP40 lysis buffer, once in SDS/DOC wash buffer, and once more in NP40 lysis buffer before being lysed in 2x Laemmli buffer, boiled, and separated on a 10% SDS-polyacrylamide gel. The immunoprecipitations used in the *in vitro* reactions described in Chapter 4 were washed twice more in PBS after the final NP40 lysis buffer wash, before being resuspended in PBS and separated into 100μL aliquots.

2.8 Polyacrylamide gel electrophoresis

Samples were solubilised by boiling in Laemmli's sample buffer and then loaded onto 10% polyacrylamide gels. Electrophoresis was carried out using an Aladin polyacrylamide gel electrophoresis running apparatus (Aladdin Enterprises Inc.). Samples were run for 900-1000 volt hours.

2.9 Western blotting

Following gel electrophoresis, samples were transferred to nitrocellulose (Scleicher & Schuell) for 3 hours at 800mA using a wet transfer apparatus (BioRad) filled with cold Towbin buffer. Nitrocellulose membranes were blocked by incubation for one hour in TBS-Tween containing either 5% bovine serum albumin for phospho-specific blots or 5% non-fat dry milk for all other blots. Membranes were then incubated overnight in blocking buffer supplemented with the indicated dilution of primary antibody. The membranes were then washed extensively in TBS-Tween prior to a one hour incubation in blocking buffer supplemented with the indicated dilution of secondary antibody. The membranes were then washed extensively in TBS-Tween and developed using ECL or SuperSignal West Pico Chemiluminescent Substrate (Pierce), depending on the sensitivity of the primary antibody. Membranes were stripped with Stripping buffer prior to reprobing with primary antibodies originating from a different species from the original primary antibody used. To increase the sensitivity of the α-Ubiquitin antibody,

membranes to be probed with this antibody were submerged under water in a glass dish and autoclaved prior to blocking and incubation with the primary antibody (Swerdlow et al., 1986).

2.10 *In vitro* piceatannol/ROS reactions

2.10.1 Protein loss reaction

One 100µL aliquot of each PBS-resuspended immunoprecipitation was used for each *in vitro* reaction condition. For each experiment, three control samples were run in conjunction with the experimental samples: untreated control, ROS alone control, and compound alone control. The ROS generation system consists of 0.02U HRP and the indicated concentration of H_2O_2. Compounds were used at the indicated concentrations. A typical reaction mixture contained the immunoprecipitated protein, a compound, HRP and H_2O_2. Reactions were begun with the addition of H_2O_2 and were terminated after 15 minutes at room temperature by the addition of 33µL of 4x Laemmli buffer. Samples were boiled for 6 minutes and 75µL of each sample was loaded on a 10% SDS-PAGE gel for Western blotting.

2.10.2 In vitro kinase assay

Lipid rafts pellets were resuspended in 1mL cold kinase assay buffer without ATP and dispensed into 50µL aliquots. Note that the $MnCl_2$ in the kinase assay buffer served to generate ROS. Piceatannol and additional treatments were added at the indicated concentrations, followed by the addition of 25µL of kinase assay buffer with or without 300µM ATP (100µM ATP final concentration). Reactions were terminated after 15 minutes at room temperature by the addition of 25µL of 4x SDS Laemmli buffer. Samples were boiled for 6 minutes and 25µL loaded on a 10% SDS-PAGE gel for Western blotting.

2.10.3 Gelatin zymography

10ng of recombinant MMP-9 was incubated in PBS with or without 2mM N-acetyl cysteine for 5 minutes on ice. Increasing concentrations of piceatannol and ROS generating compounds, either 10mM $MnCl_2$ or 100µM H_2O_2 and 0.02U of HRP, were then added to the reactions. After a 10 minute incubation at room temperature, the reactions were terminated by the addition of 4x Zymography loading buffer. Samples

were loaded on a 12% SDS-PAGE gel embedded with 1mg/mL gelatin. The gel was run for 1000Vh, washed for 15 minutes in Rinse buffer, and then washed in fresh Rinse buffer overnight. The zymography gel was thoroughly rinsed in distilled water and placed in a clean dish with Incubation buffer overnight at 37°C. After the overnight incubation, the zymography gel was rinsed with distilled water, stained with Coomassie stain for 1 hour, and then destained overnight in Destain. Destained zymography gels were documented using an HP ScanJet 4C.

2.10.4 DNA damage assay

The ability of piceatannol-like compounds to induce single-strand and double-strand breaks was determined using methodology based on (Subramanian et al., 2004). *In vitro* reactions were made up in HBSS and contained 200ng of pSP72 plasmid, 50µM compound and 50µM of the indicated metal chloride. Reactions were incubated at 37°C for 1 hour, were stopped by the addition of 6x gel loading buffer, and were subsequently loaded onto a 1% agarose gel with ethidium bromide. DNA was visualized and documented using a BioRad Gel Doc 2000 and the Quantity One 4.0.3 software.

2.10.5 Spectrophotometric detection of o-benzoquinones

The increased absorbance of o-benzoquinones at 400nm was used to detect the presence of this chemical species (Sugumaran et al., 1992). 100µL aliquots of ddH_2O containing 500µM piceatannol-like compound, 0.08ng of HRP-conjugatged IgG, 500µM H_2O_2, with or without 500µM N-acetyl cysteine, were prepared and their absorbance at 400nm was monitored every 5 seconds for 2.5 minutes using a Beckman DU530 UV/Vis spectrophotometer. The change in A_{400} at each time point was calculated by subtracting the A_{400} of the aliquot before the addition of H_2O_2 from the A_{400} at the specific time point. These ΔA_{400} values were then plotted over time to determine if the addition of ROS, produced by HRP and H_2O_2, induced the piceatannol-like compounds to form o-benzoquinones.

2.11 RT-PCR

70ZCbl expressing murine 3T3 fibroblasts were plated at $5x10^5$ cells per well the day prior to treatment. The next day cells were treated with 200µM piceatannol or DMSO for 1, 3, 8, and 24 hours. RNA was isolated with TRIzol reagent (Invitrogen)

using the manufacturers protocol. RNA was then DNase treated with 0.5 units of RQ1 RNase free DNase (Promega) in the presence of 20 units RNase Block (Invitrogen) for 30 minutes at 37°C. RNA was phenol:chloroform extracted and 2µg of RNA was used to make cDNA using Superscript II (Invitrogen) following the manufacturers protocol. RT-PCR was performed using the primer drop method (Wong et al., 1994) using the Cbl4 (Cbl4: 5'-GCCCTGACCTTCTGA TTCCTGCCA-3') and MiddleCblF (MiddleCblF: 5'-GATTGATGGCTTCAGGGAAGG-3') primers to detect c-Cbl/70ZCbl and the mGAPDH-F (mGAPDH-F: 5'-ACCACAGTCCATGCCATCA C-3') and mGAPDH-R (mGAPDH-R: 5'-TCCAC CACCCTGTTGCTGTA-3') primers to detect GAPDH. RT-PCR reactions were diluted with 6X DNA loading buffer and loaded onto a 1% agarose gel with ethidium bromide. Products were visualized and documented using a BioRad Gel Doc 2000 and the Quantity One 4.0.3 software.

2.12 Flow cytometry

2.12.1 Analysis of apoptosis

$1x10^6$ 70Z/3 cells in 1 mL media were treated with DMSO or varying concentrations of piceatannol for 3 or 8 hours. Detection of piceatannol-induced apoptosis in 70Z/3 cells by flow cytometry was accomplished using the ApoAlert Annexin V-FITC Apoptosis Kit (BD Biosciences) as per the manufacturer's protocol.

2.12.2 Analysis of cathepsin B/L activity

Flow cytometric analysis of cathepsin activity was performed using a stock solution of 5mM $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 (Molecular Probes; Eugene, OR) in DMSO. Mouse fibroblast cells stably overexpressing 70ZCbl were plated in 6 well dishes, $7.5x10^5$ cells per well. The following day, cells were treated for 4 hours with lysosomal inhibitors. Cells were trypsinized, resuspended in 10 mL of media, and washed once in 1mL HBS-E (5mM HEPES pH 7.35, 150mM NaCl, 2mM EDTA). Cells were resuspended in 250µL of HBS-E containing 10µM $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 and lysosomal inhibitor at the same concentration used to treat the cells. Cells were then incubated at 37°C/ 5% CO_2 for 20 minutes. Cell suspensions were diluted to 500µL with 225µL HBS-E and 25µL of 50µg/mL propidium iodide (Sigma) and incubated at room temperature for 5 minutes. Cells were then analyzed by flow cytometry using an

excitation wavelength 488nm and reading $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 at an emission wavelength of 525nm and propidium iodide at an emission wavelength of 633nm. Propidium iodide positive cells were subtracted from the $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 positive cells for the determination of relative cathepsin activity. The average means of $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 intensity of 5 experiments were normalized to their respective untreated controls. Values were then represented as relative percentages of normal cathepsin activity.

Chapter Three: Cbl-Mediated Cellular Transformation

3.1 Introduction

3.1.1 The Cbl family of proteins

The Cbl family of proteins are the cellular homologues of the oncogenic, virally derived, v-Cbl. These proteins are named for the retrovirus from which v-Cbl was cloned, the Cas NS-1 retrovirus, also known as the Casitas B-lineage lymphoma virus, due to its propensity to induce pre-B-cell lymphomas in mice (Langdon et al., 1989). In mammals there are three Cbl family members: c-Cbl, Cbl-b, and Cbl-c (also known as Cbl-3). Cbl-b and c-Cbl are expressed in many different cell types, with the highest expression levels in cells of hematopoietic origin (Langdon et al., 1989; Keane et al., 1995). In contrast, the expression of the smallest Cbl family member, Cbl-c, is restricted to epithelial cells of the epidermis, the gastrointestinal tract and the respiratory tract, as well as the urinary and reproductive systems (Keane et al., 1999; Kim et al., 1999).

In these various tissues, the Cbl-family proteins serve as negative regulators of cellular signalling, due to their E3 ubiquitin-ligase activity. This activity is localized to Cbl's conserved amino-terminal region, which includes a tyrosine kinase binding domain (TKB), a Linker domain, and a RING finger domain (Figure 3.1) (Swaminathan and Tsygankov, 2006). Each of these domains is important in enabling Cbl proteins to function as E3 ubiquitin-ligases. Proteins that are to be ubiquitinated by Cbl are recognized, in part, through Cbl's TKB domain. The TKB domain recognizes specific phosphorylated tyrosine residues on these target proteins, thereby providing specificity to the ubiquitination process. A number of different PTKs are targeted for degradation by the TKB domain, including EGFR, PDGFR, Syk, and Zap-70 (Thien and Langdon, 2001; Swaminathan and Tsygankov, 2006). Several adaptor and regulatory proteins are also targeted for ubiquitination by the TKB domain; including, the B cell adaptor protein, BLNK (Yasuda et al., 2000), the insulin receptor-associated adaptor protein, APS (Yokouchi et al., 1999), as well as Sprouty, an inducible antagonist of Cbl's E3-ligase activity (Rubin et al., 2003). Phosphorylation of tyrosine residues in Cbl's Linker domain regulates the E3 ubiquitin-ligase activity mediated by the RING finger domain (Kassenbrock and Anderson, 2004). Upon activation, the RING finger domain efficiently

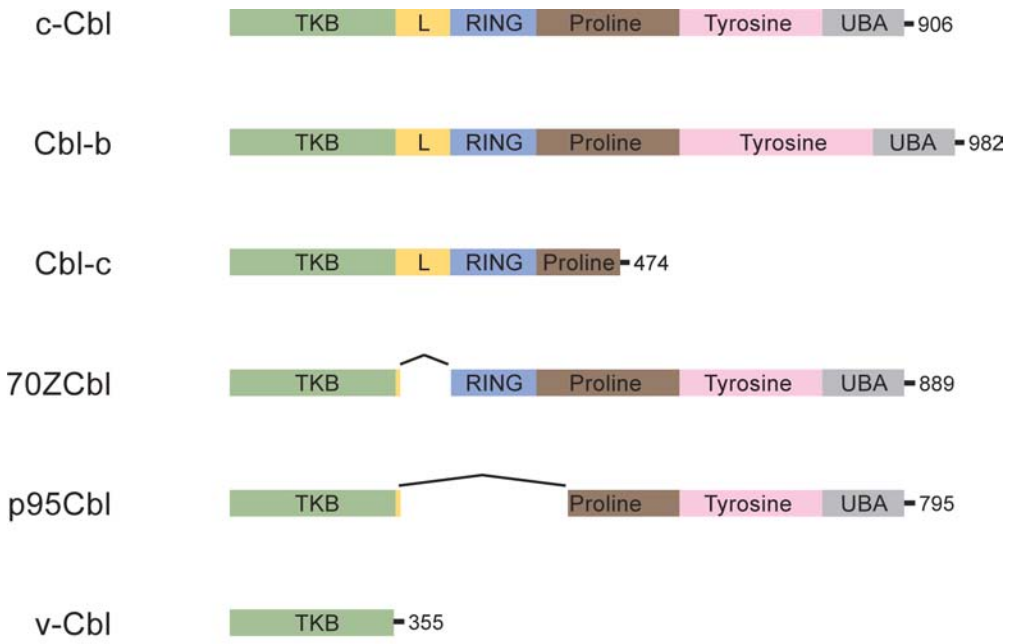


Figure 3.1 – Schematic representation of the domain structure of the Cbl family of proteins and their naturally occurring transforming mutants.

c-Cbl and Cbl-b each possess tyrosine kinase binding domains (TKB), Linker domains (L), RING finger domains, Proline-rich regions (Proline), Tyrosine-rich regions (Tyrosine) and ubiquitin association motifs/ leucine zippers (UBA). The shorter Cbl-c possesses the conserved Cbl amino-terminus, but is truncated relative to c-Cbl and Cbl-b and only possesses a small Proline-rich region. 70ZCbl and p95Cbl are c-Cbl mutants that possess internal deletions of 17 and 111 amino acids, respectively. The v-Cbl protein is truncated at amino acid 355, and only possesses a TKB domain.

catalyzes the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to the target protein. Ubiquitination of target proteins can result in mono-, multi-, and poly-ubiquitination leading to endocytosis, lysosomal degradation, and proteasomal degradation, respectively (Thien and Langdon, 2001; Thien and Langdon, 2005; Swaminathan and Tsygankov, 2006). In addition to Cbl's negative regulatory function, this protein is also known to act as a positive regulator in cellular signalling processes by serving as an adaptor protein (Swaminathan and Tsygankov, 2006).

Cbl family members play positive regulatory roles as adaptor proteins in cellular signalling events due to their conserved carboxy-terminal regions, which contain several protein-protein interaction motifs; including, a Proline-rich region, a Tyrosine-rich region and a leucine zipper/ubiquitin association motif (LZ/UBA) (Figure 3.1) (Swaminathan and Tsygankov, 2006). Each of these regions is important in enabling Cbl-proteins to act as multifunctional adapter proteins by helping to recruit effector proteins to activated receptor signalling complexes (Bonita et al., 1997). The Proline-rich region adjacent to the RING finger domain serves as a docking site for SH3 domain containing proteins, including Grb2 and several Src family kinases (Schmidt and Dikic, 2005). Tyrosine residues that are situated in the extreme carboxy-terminus of the Cbl, when phosphorylated, serve as docking sites for SH2 domain containing proteins. These proteins include the p85 subunit of PI3K, Crk, and Vav (Schmidt and Dikic, 2005). Cbl's LZ/UBA motif has been shown to self-associate and as such, to enable c-Cbl and Cbl-b to form both homo- and hetero-dimers (Liu et al., 2003). Together, the Proline-rich region, the Tyrosine-rich region, and the LZ/UBA enable Cbl to participate in the formation of multiprotein signalling complexes, involved in the transduction of signals from FcReceptors, the T cell receptor, the B cell receptor, G protein coupled receptors and RTKs (Swaminathan and Tsygankov, 2006).

3.1.2 The TKB domain

Cbl family proteins show a high degree of sequence similarity in their amino-terminal regions. This region folds into a phosphotyrosine binding unit termed the tyrosine kinase binding domain (TKB) (Figure 3.1). This unique domain is comprised of a four-helix bundle, a Ca^{2+} binding EF hand, and a variant SH2 domain (Meng et al.,

1999). The foremost function of the TKB domain is to help determine Cbl's substrate specificity by engaging specific phosphorylated tyrosine residues on activated substrates through its variant SH2 domain. The most extensively studied of Cbl's substrates are RTKs, such as the EGFR and PDGFR, as well as NRTKs, such as Syk and Zap-70. X-ray crystallography of the c-Cbl TKB domain complexed to a phosphopeptide representing its binding site in ZAP-70, has demonstrated that the SH2-variant region of the TKB domain binds phosphorylated tyrosine residues in a manner similar to other SH2 domains (Meng et al., 1999). However, unlike SH2 domains, the TKB domain is not thought to promote a strong association with its binding partners and is currently believed to require the assistance of Cbl-associated adaptor proteins to solidify these interactions. As an example, the Grb2 adaptor protein, which binds to Cbl through SH3 domain-Proline rich region interactions, helps Cbl to associate with RTKs. This is evidenced by mutants of EGFR and MetR that are unable to bind Cbl's TKB domain and are still ubiquitinated and downregulated by Cbl in a Grb-2 dependent manner (Waterman et al., 2002; Li et al., 2007). Despite the dispensability of the TKB domain's function in RTK ubiquitination, the presence of a functional TKB domain-RTK interaction does increase the efficiency of this process (Waterman et al., 2002; Li et al., 2007). This suggests that the TKB domain may play an important structural role in orienting the RING finger domain in relation to the substrate, increasing ubiquitination efficiency (Zheng et al., 2000).

TKB domain-mediated interactions are not only mediated by the SH2-variant region of this domain; the four-helix bundle has also been shown to associate with tyrosine phosphorylated proteins (Hu and Hubbard, 2005). In addition, the TKB domain has been implicated in mediating Cbl's association with and stabilization of microtubules. This interaction was shown to be independent of the TKB domain's variant-SH2 region and is thought to be independent of tyrosine phosphorylation (Teckchandani et al., 2005).

Thus, unlike classical SH2 domains, Cbl's highly conserved TKB domain promotes phosphotyrosine-dependent and -independent protein binding, non-classical phosphotyrosine binding through its four-helix bundle, and has potentially important

roles in promoting E3 ubiquitin-ligase activity through the orientation of Cbl's RING finger with respect to its substrates.

3.1.3 The Linker domain

The Linker domain separates the TKB domain from the RING finger domain. It is composed of a highly conserved stretch of amino acids that form an α-helix (Zheng et al., 2000) (Figure 3.1). The TKB domain makes intermolecular contacts, through its SH2 variant region and EF hand, with the Linker domain centered on the conserved tyrosine residues Y368 and Y371 of the α-helix, in human c-Cbl (Zheng et al., 2000). These Linker domain tyrosine residues have been shown to play an important role in the regulation of Cbl's E3 ubiquitin-ligase activity and substrate association (Andoniou et al., 1994; Levkowitz et al., 1999; Kassenbrock and Anderson, 2004). Mutation of Y371 to phenylalanine abolishes Cbl's E3-ligase activity and decreases the TKB domain's affinity for tyrosine phosphorylated EGFR, whereas mutation of Y371 to a phosphomimetic glutamate residue constitutively promotes E3 ubiquitin-ligase activity and increases the TKB domain's affinity for tyrosine phosphorylated EGFR (Levkowitz et al., 1999; Kassenbrock and Anderson, 2004). E3 ubiquitin-ligase activity is further increased when both Y368 and Y371 are mutated to phosphomimetic glutamate residues (Kassenbrock and Anderson, 2004). Molecular modelling data predicts that the phosphorylation of these tyrosine residues would result in significant changes to the orientation of the Linker domain α-helix, altering its association with both the TKB and RING finger domains and altering the interaction between these domains and E2 ubiquitin-conjugating proteins (Zheng et al., 2000). Similar to the phosphomimetic mutations of Y368 and Y371, *in vitro* phosphorylation of these two residues induces a conformational change in the TKB domain that promotes its association with tyrosine phosphorylated EGFR and that also enhances Cbl's E3 ubiquitin-ligase activity (Kassenbrock and Anderson, 2004).

These data imply that phosphorylation of the Linker domain, potentially by Cbl's many PTK substrates, induces a conformational change that affects the regulation of the ubiquitination of Cbl substrates. This is supported by the point deletion of Y368 or Y371 in the Linker domain: these mutations are predicted to disrupt the α-helical nature of the Linker domain and have been shown to abrogate Cbl's E3 ubiquitin-ligase activity *in vivo*

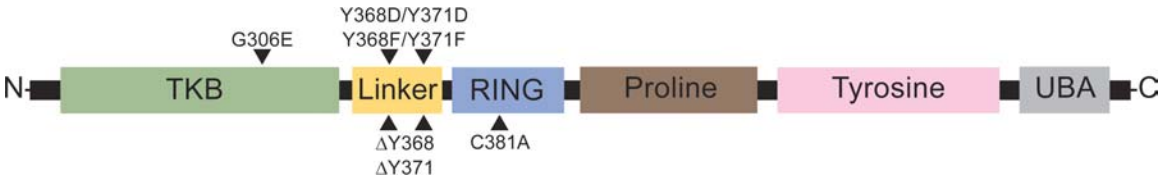
(Andoniou et al., 1994; Thien et al., 2001). These point deletion mutations, presumably by leading to the loss of E3 ubiquitin-ligase activity, render Cbl oncogenic (Andoniou et al., 1994; Thien et al., 2001). Interestingly the Y371F mutation, which also abolishes E3 ubiquitin-ligase activity but is not predicted to disrupt the Linker domain structure, does not render Cbl oncogenic (Andoniou et al., 1994; Levkowitz et al., 1999) (Figure 3.2). This indicates that the Linker domain's ability to regulate Cbl's conformation is integral to Cbl's ability to maintain cellular homeostasis.

3.1.4 The RING finger domain

RING finger domains are protein-protein interaction domains of approximately 70 amino acids that feature a set of distinctively spaced cysteine and histidine residues. These residues are required to interact with two zinc ions and thereby stabilize the characteristic globular shape of the RING figure domain (Pickart, 2001). Cbl family proteins contain a highly conserved C3H4 RING finger domain, which is named for the number of constitutent cysteine and histidine residues. This RING finger domain allows Cbl family proteins to function as E3 ubiquitin-ligases by mediating the transfer of ubiquitin from E2 ubiquitin-conjugating enzymes to their substrate proteins (Figure 3.1). Mutation of the Zn^{+2} binding residues, as is seen in the C381A mutation, is predicted to disrupt the RING structure and has been shown to abrogate E3 ubiquitin-ligase activity (Joazeiro et al., 1999; Pickart, 2001).

RING finger E3 ubiquitin-ligases are thought to act as scaffolds for the transfer of Ub from E2 enzymes to substrate proteins (Pickart, 2001) and have no catalytic activity of their own. The idea that c-Cbl is simply required to position the E2 active site, which contains a thiol-conjugated ubiquitin molecule, is supported by the fact that c-Cbl's RING finger binds the E2 protein at a site more than 15Å distal to the site of activity (Zheng et al., 2000).

More recently, Ozkan and colleagues have demonstrated that the interaction between the RING finger E3 proteins and the E2 ubiquitin-conjugating enzymes induces the allosteric activation of active site residues in the E2 protein, enhancing its ability to transfer ubiquitin to target substrates (Ozkan et al., 2005). This allosteric activation of ubiquitin-ligase activity was not accompanied by any gross structural changes in either



Mutation	Effect
C381A	Abrogates E3 ubiquitin-ligase activity
G306E	Prevents TKB domain from associating with tyrosine phosphorylated binding partners
ΔY368/ΔY371	Promotes cellular transformation, abrogates E3 ubiquitin-ligase activity *in vivo* but not *in vitro*
Y368D/Y371D	Activates the TKB domain, increasing its affinity for its binding partners, and increases E3 ubiquitin-ligase activity *in vitro*
Y368F/Y371F	Blocks the activation of the TKB domain and reduces E3 ubiquitin-ligase activity *in vivo* and *in vitro*

Figure 3.2 – Schematic representation of c-Cbl, including a tabular summary of the mutations used and their consequences to normal c-Cbl function.

enzyme. This led the authors to conclude that the interaction between the E3- and E2-enzymes induced subtle changes in the energetic coupling between several important amino acids within the E2-enzyme that facilitate the transfer of ubiquitin (Ozkan et al., 2005).

Regulation of Cbl's E3 ubiquitin-ligase activity is mediated in *trans* by binding to Sprouty proteins (Rubin et al., 2003). The Sprouty family of proteins are the only other proteins known to bind to Cbl's RING finger domain other than E2 ubiquitin-conjugating enzymes (Rubin et al., 2003). Sprouty proteins bind to the RING finger domain and prevent it from associating with E2 ubiquitin-conjugating enzymes thereby negatively regulating Cbl's E3 ubiquitin-ligase activity (Rubin et al., 2003). Tyrosine phosphorylation of Sprouty leads to its release from the RING finger domain and to its subsequent association with Cbl's TKB domain (Mason et al., 2004). Sprouty binding to the TKB domain antagonizes Cbl's TKB domain-mediated RTK association and also induces Cbl-mediated Sprouty ubiquitination (Rubin et al., 2003). Therefore, Sprouty negatively regulates Cbl's ability to ubiquitinate cellular signalling molecules by blocking its association with E2 enzymes and also inhibits Cbl's ability to associate with and nucleate multi-protein signalling complexes by blocking its TKB domain-mediated association with activated RTKs.

Cbl's E3 ubiquitin-ligase activity is also regulated in *cis* by the Linker domain (Kassenbrock and Anderson, 2004). As mentioned, tyrosine phosphorylation of Cbl's Linker domain induces a conformational change that leads to an increase in E3 ubiquitin-ligase activity (Kassenbrock and Anderson, 2004). Considering the recent evidence suggesting that binding of RING finger E3 proteins to E2 enzymes induces a conformationaly subtle allosteric activation of the E2 enzyme, presented by Ozkan and colleagues (Ozkan et al., 2005), it is possible that the tyrosine phosphorylation of CblY371 and CblY368 is directly involved in this process. Structural studies examining the interaction between the E2 enzyme, UbcH7, and c-Cbl have shown that two negatively charged glutamate residues in the Linker domain, CblE366 and CblE369, are involved in forming salt bridges with two positively charged arginine residues in UbcH7, R5 and R15 (Zheng et al., 2000). The increase in negative charge, introduced by the

phosphorylation of CblY368 and CblY371, may subtly alter the interaction between the Linker domain and the arginine residues enough to achieve the allosteric activation of UbcH7 by c-Cbl. Alternatively, the Linker domain-induced conformational change may simply alleviate TKB domain-mediated negative regulation of the RING finger domain by disrupting the interaction between the four-helix bundle and the RING finger domain (Zheng et al., 2000). Regulation of Cbl's E3 ubiquitin-ligase activity may also be mediated through the protein-protein interactions facilitated by its carboxy-terminal adaptor domains.

3.1.5 The Proline-rich region

Adjacent to the RING finger domain is a stretch of amino acids collectively termed the Proline-rich region. This region manifests the largest divergence in domain structure between the three Cbl family members: Cbl-3 has a relatively short Proline-rich region, containing 5 potential proline-rich SH3 domain-binding sites, whereas c-Cbl and Cbl-b have 15 and 17 SH3 domain-binding sites, respectively (Figure 3.1) (Swaminathan and Tsygankov, 2006). The large number of SH3 domain-binding sites has been shown to mediate protein-protein interactions with many adaptor and effector proteins. For example, PXXP motifs in the Proline-rich region have been shown to interact with the SH3 domains of several adaptor proteins; including, Grb2, Nck, and FRS2 (Schmidt and Dikic, 2005). Several of these interactions have been shown to be constitutive and are important for Cbl's normal function: in the absence of Grb2-mediated RTK binding, Cbl is not appropriately tyrosine phosphorylated in response to RTK stimulation and therefore Cbl does not mediate appropriate RTK ubiquitination and downregulation (Sun et al., 2007). This Proline-rich region also mediates interactions with a number of NRTKs including several SFKs, c-Abl, Btk, Itk, and Tyk2 (Schmidt and Dikic, 2005). Many of these NRTKs, through their association with Cbl, are also targeted for ubiquitination (Swaminathan and Tsygankov, 2006), although there is evidence supporting the additional involvement of the TKB domain in this process (Yokouchi et al., 2001).

3.1.6 The Tyrosine-rich region

Three main tyrosine residues in the carboxy-terminus of Cbl become tyrosine phosphorylated in response to RTK activation, and serve to recruit a number of important SH2 domain containing signalling effector proteins (Figure 3.1). Phosphorylation of these residues, *in vivo*, is critical for the recruitment of several important effectors proteins; including, the p85 sub-unit of PI3K through Y731, the Rac-GEF Vav through CblY700, and the adaptor proteins Crk, CrkII, and CrkL through CblY700 and CblY774 (Andoniou et al., 1996; Feshchenko et al., 1999). Notably, many of the proteins that associate with the carboxy-terminus are involved in Rac activation. Confirming the importance of this region in mediating Rac activation, c-Cbl truncation mutants have been shown to promote defects in RTK-induced actin remodelling, preventing the formation of actin lamellae, lamellipodia, and membrane ruffles (Scaife and Langdon, 2000). These residues are differentially phosphorylated by a number of different NRTKs, all of which are known to associate with Cbl through TKB, SH2, and/or SH3 domain-mediated interactions. This differential phosphorylation likely produces the specificity required for Cbl proteins to participate in the formation of context specific multiprotein signalling complexes. However, most of the studies concerning Cbl-tyrosine phosphorylation have been performed *in vitro* with purified kinases or kinase domains; therefore, it is unclear if they are representative of what occurs *in vivo* (Swaminathan and Tsygankov, 2006).

In addition to the phosphorylation of the carboxy-terminal tyrosine residues, interactions between SH2 domain-containing proteins and the Tyrosine-rich region of Cbl may also be regulated by Cbl's Linker domain-mediated conformational change. Phosphorylation of the Linker domain CblY371 has been shown to be required for c-Cbl's interaction with PI3K and Crk (Miura et al., 2003; Standaert et al., 2004). The position of this tyrosine residue between two large globular domains, the TKB domain and the RING finger domain, suggests the phosphorylation of this amino acid is not directly involved in protein-protein interactions with these two proteins as it would not be physically accessible to either of these proteins. Rather, it suggests that the

conformational change induced by the phosphorylation of CblY371 alleviates previous structural impediments to PI3K and Crk binding.

3.1.7 The Leucine zipper motif/Ubiquitin association motif

Superimposed at the extreme carboxy-terminus of Cbl are a leucine zipper motif and a ubiquitin-association motif (UBA) (Figure 3.1). While the structural elements of these motifs overlap, their functions can be disassociated (Bartkiewicz et al., 1999; Liu et al., 2003; Davies et al., 2004). The leucine zipper has been shown to mediate homo-and hetero-dimerization of Cbl proteins (Bartkiewicz et al., 1999; Liu et al., 2003), whereas the UBA, as its name suggests, is involved in binding to ubiquitin (Davies et al., 2004). The involvement of these C-terminal motifs in the function of Cbl is unclear, since the UBA domain is dispensable for Cbl-mediated ubiquitination (Levkowitz et al., 1999) and dimerization of Cbl is not absolutely required for its association with its binding partners (Liu et al., 2003).

3.1.8 Cbl-mediated regulation of RTK signalling

Of the Cbl family proteins, c-Cbl has been the most extensively studied and the mechanism determined for its function is thought to apply to Cbl-b and Cbl-3 due to the high degree of similarity between these proteins (Swaminathan and Tsygankov, 2006) (Figure 3.3). Cbl resides in the cytosol of resting cells, possibly associated with microtubules through phosphotyrosine-independent TKB domain-mediated interactions (Swaminathan and Tsygankov, 2006). Upon activation of receptor tyrosine kinases by ligand binding, c-Cbl is recruited from the cytosol to the activated receptor complex in association with Grb2. Cbl is constitutively associated with Grb2 via the interaction between Cbl's Proline-rich region and Grb2's SH3 domain (Jiang et al., 2003; Jiang and Sorkin, 2003). This Grb2-dependent recruitment of Cbl into proximity with the activated RTKs leads to its tyrosine phosphorylation. This tyrosine phosphorylation induces a conformational change in Cbl's Linker domain, which subsequently alters the conformation of the TKB domain, allowing for stronger associations between Cbl's TKB domain and the tyrosine phosphorylated RTKs (Kassenbrock and Anderson, 2004). Membrane-associated Cbl then facilitates signal propagation through its recruitment of additional signalling effector molecules, which is mediated by its many protein-protein

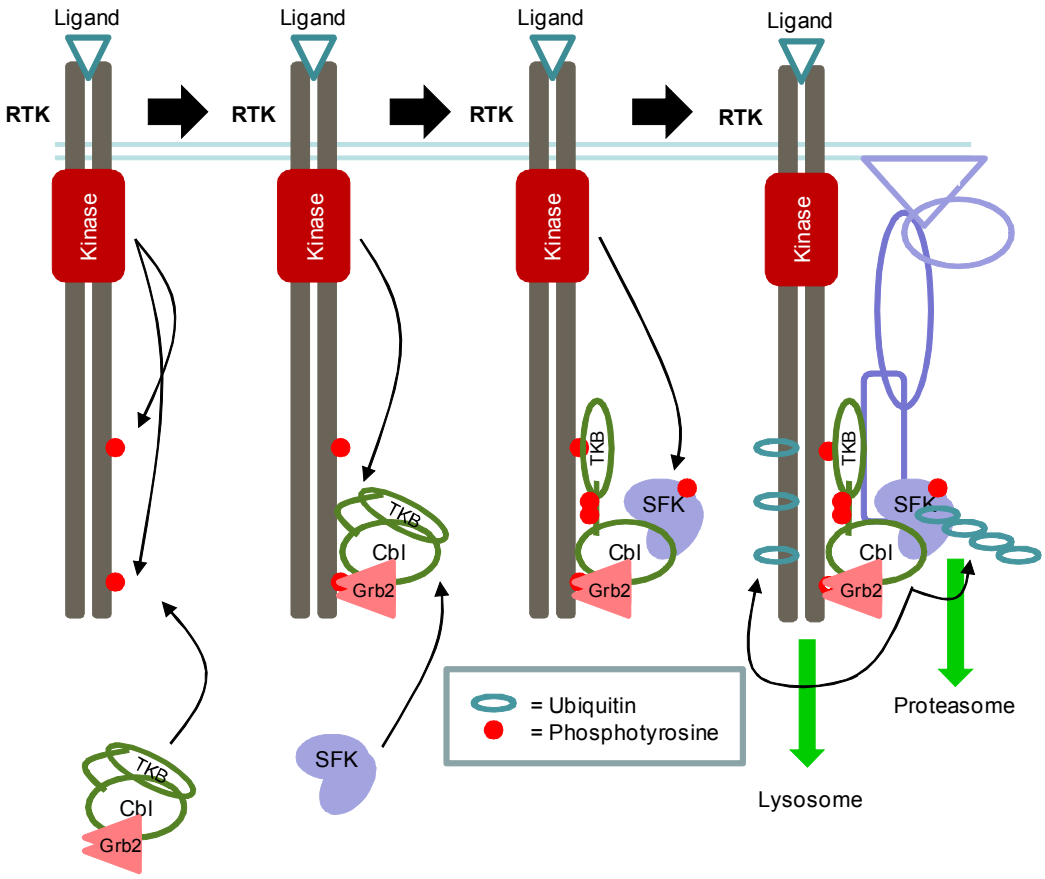


Figure 3.3 – Schematic representation of Cbl's normal function using RTK signalling as an example.

Autophosphorylation of activated RTKs recruits the Grb2-Cbl complex in an SH2 domain-dependent manner. Localization of Cbl proximal to the RTK leads to the tyrosine phosphorylation of the Linker domain, inducing the activation of the TKB domain. Phosphorylation of Cbl's Tyrosine-rich region also leads to the recruitment of signalling effector molecules such as Src-family kinases (SFK). Tyrosine phosphorylation of Cbl's Linker domain also induces its E3 ubiquitin-ligase activity, promoting the multi-ubiquitination of the associated RTK and the poly-ubiquitination of the associated signalling effectors. The multi-ubiquitination of the RTK, in conjunction with Cbl's recruitment of CIN85 and endophilin, promote the endocytosis and subsequent lysosomal degradation of the RTK. The poly-ubiquitination of the signalling effectors leads to their recognition and degradation by the 26S proteasome.

interaction motifs (Swaminathan and Tsygankov, 2006). Tyrosine phosphorylation of the Linker domain also serves to activate Cbl's E3-ubiquitin ligase activity (Kassenbrock and Anderson, 2004), allowing Cbl to multi-ubiquitinate the associated RTKs and poly-ubiquitinate the associated signalling effector proteins. This multi-ubiquitination of RTKs leads to their association with a number of UBA motif containing adaptor proteins involved in regulating clathrin-coated pit formation (Thien and Langdon, 2005; Swaminathan and Tsygankov, 2006). The subsequent movement of RTKs into clathrin-coated pits allows for their efficient endocytosis and facilitates signal termination. On the other hand, the poly-ubiquitination of NRTKs and other cytosolic signalling effector molecules leads to signal termination via their recognition and subsequent degradation by the 26S proteasome (Thien and Langdon, 2001; Thien and Langdon, 2005; Swaminathan and Tsygankov, 2006).

Although the ubiquitination of RTKs is important for signal termination, surprisingly, Cbl's E3 ubiquitin-ligase activity has been shown to be dispensable for RTK internalization (Swaminathan and Tsygankov, 2006). Therefore one might expect that Cbl function in this process is redundant. However, considerable evidence does favour the existence of a second mechanism by which c-Cbl and Cbl-b can mediate RTK downregulation, independent of E3 ubiquitin-ligase activity. This mechanism is dependent upon their interaction with the SH3 domain-containing protein CIN85, which binds to an atypical SH3-interacting motif (PXXXPR) found in the carboxy-terminal region of c-Cbl and Cbl-b (Szymkiewicz et al., 2002; Kowanetz et al., 2003; Kurakin et al., 2003). CIN85 is constitutively associated with endophilin, a regulatory component of clathrin-coated pit formation. Therefore, the interaction of CIN85 with Cbl helps to localize Cbl and associated proteins, such as RTKs, to clathrin-coated pits for endocytosis. Evidence that supports this mechanism shows that the use of a dominant negative CIN85 protein, or the mutation of Cbl's PXXXPR motif, blocks the endocytosis of activated EGFR without affecting Cbl's ability to mediate EGFR ubiquitination (Soubeyran et al., 2002; Kowanetz et al., 2003). Met receptor has also been shown to be regulated by c-Cbl in a similar manner (Petrelli et al., 2002). While these data argue that the PXXXPR motif, and not E3 ubiquitin-ligase activity, is required for Cbl-mediated

RTK downregulation, this argument is refuted by other evidence. Several reports have indicated that truncated versions of Cbl, which can no longer bind CIN85, are still able to mediate EGFR internalization (Keane et al., 1999; Levkowitz et al., 1999; Lill et al., 2000). As well, transforming mutants of Cbl that contain an intact PXXXPR motif, but have lost their E3 ubiquitin-ligase activity, are unable to effectively mediate RTK downregulation (Swaminathan and Tsygankov, 2006). Huang and Sorkin performed experiments to evaluate these contradictory reports by fusing the amino-terminus of Cbl, containing the RING finger domain, or the carboxy-terminus of Cbl, containing the PXXXPR motif, with the SH2 domain of Grb2 (Huang and Sorkin, 2005). They found that when they expressed these proteins in Grb2 depleted cells, the SH2 domain of Grb2 was able to mediate the association of both of these Cbl fusion proteins with EGFR and that both the RING finger domain and the PXXXPR motif were able to mediate EGFR internalization. However, although both fusion proteins were able to induce EGFR internalization, the presence of the RING finger domain elicited a significantly greater EGFR-internalization response than the presence of the PXXXPR motif (Huang and Sorkin, 2005). Altogether, these studies emphasize the complexity of the Cbl-mediated RTK internalization process, which can be controlled by ubiquitination-dependent and -independent mechanisms.

3.1.9 Transforming mutants of Cbl

Cbl was originally identified as a homologue of the viral oncogene v-Cbl (Langdon et al., 1989). The v-Cbl protein is composed of the first 355 amino acids of c-Cbl, which comprise the TKB domain (Langdon et al., 1989). Two other naturally occurring transforming mutants of Cbl have also been identified: 70ZCbl and p95Cbl (Andoniou et al., 1994; Bisson et al., 2002). 70ZCbl and p95Cbl contain internal deletions that encompass the Linker domain as well as part, or all, of the RING finger domain (Figure 3.1). These transforming Cbl-mutants have lost their E3 ubiquitin-ligase activity and are thought to mediate cellular transformation *via* a dominant-negative mechanism: E3 ubiquitin-ligase null, transforming Cbl-mutants compete for E3 ubiquitin-ligase substrates with wtCbl, allowing these substrates to escape ubiquitination and signal termination, thereby inducing transformation. In support of this model, the artificially

generated Linker domain point-deletion mutants, CblΔY368 and CblΔY371, (Figure 3.2) have also lost their *in vivo* E3 ubiquitin-ligase activity and are also transforming (Thien et al., 2001). This dominant-negative competition model of Cbl-mediated transformation is also supported by the dependence of Cbl-mediated transformation on a functional TKB domain. Cbl's TKB domain is responsible for Cbl's association with E3 ubiquitin-ligase substrates. The introduction of a G306E mutation (Figure 3.2) into the TKB domain of Cbl prevents this association with E3 ubiquitin-ligase substrates. The introduction of a G306E mutation into the transforming Cbl-mutants prevents cellular transformation (Bonita et al., 1997), thereby demonstrating that the loss of E3 ubiquitin-ligase activity is only sufficient to result in transformation in the dominant negative competition model. Therefore, while all of the transforming mutants of Cbl have lost their *in vivo* E3 ubiquitin-ligase activity, the loss of E3 ubiquitin-ligase activity is not sufficient to lead to cellular transformation.

This dominant negative model of Cbl-mediated transformation is probed by two artificial mutations of Cbl. The CblY371F Linker domain point mutation and the CblC381A RING finger point mutation, have lost their *in vivo* E3 ubiquitin-ligase activity but do induce robust cellular transformation (Andoniou et al., 1994; Thien et al., 2001). The CblY371F mutant, despite its loss of E3 ubiquitin-ligase activity and its inability to induce cellular transformation, still fits within the competition model of Cbl-mediated transformation. The CblY371F mutation prevents the phosphorylation of Cbl's Linker domain and therefore prevents the phosphorylation-induced activation of Cbl's TKB domain. Kassenbrock and Anderson demonstrated that CblY371F, due to its inability to activate the TKB domain, is defective at associating with activated EGFR (Kassenbrock and Anderson, 2004). Thus, this mutant of Cbl does not violate the competitive dominant-negative model of Cbl-mediated transformation: the inability of this E3 ubiqutin-ligase null protein to transform cells is explained by its inability to compete with wtCbl for E3 ubiquitin-ligase substrates due to its defective TKB domain activation. CblC381A, on the other hand, does not fit within the competitive dominant negative model of Cbl-mediated transformation. This Cbl mutant has no E3 ubiquitin-ligase activity, is competent to associate with proteins through its TKB domain, but does

not induce cellular transformation (Thien et al., 2001). To accommodate this data, the current dominant negative model for Cbl-mediated transformation must be revised.

I propose a new model for Cbl-mediated transformation, which can explain the inability of the C381A E3 ubiquitin-ligase deficient Cbl-mutant to transform cells (Figure 3.4). This model is based on the fact that transforming mutants of Cbl are constitutively tyrosine phosphorylated and membrane associated (Swaminathan and Tsygankov, 2006) and also on the recent observation that the Linker domain induced conformational change increases the ability of Cbl's TKB domain to associate with activated RTKs (Kassenbrock and Anderson, 2004). I propose that the disruption of the Linker domain α-helix *via* deletion mutations, similar to the deletion mutations found in the naturally occurring and artificially generated transforming Cbl mutants, forces Cbl to adopt a conformation similar to that induced by the phosphorylation of the Linker domain (Kassenbrock and Anderson, 2004). Similar to what has been observed with the Linker domain phosphorylation-dependent conformational change this mutation-induced conformational change would therefore activate the TKB domain and increase its affinity for target proteins (Kassenbrock and Anderson, 2004). However, unlike the phosphorylation-induced conformational change, this mutation-induced conformational change cannot be reversed by phosphatase activity and is thus permanent and unregulatable. In addition, since the Linker domain has been shown to be involved in Cbl's association with E2 ubiquitin-conjugating enzymes (Zheng et al., 2000), I propose that the conformationally disruptive Linker domain deletion mutations also abrogate E3 ubiquitin-ligase activity, regardless of whether they include part, or all, of the RING finger domain. As a result, mutations that affect the integrity of Cbl's Linker domain generate potent dominant negative Cbl proteins by abrogating E3 ubiquitin-ligase activity and by conferring a "competitive advantage" to these mutant proteins by increasing their TKB domain's affinity for its binding partners. This model explains how mutations that abrogate E3 ubiquitin-ligase activity are not necessarily transforming. CblC381A is not transforming as this Cbl mutant binds to RTKs in a regulated manner and therefore has no competitive advantage over wtCbl. CblY371F is not transforming as this mutant cannot be activated by Linker domain tyrosine phosphorylation and therefore its TKB

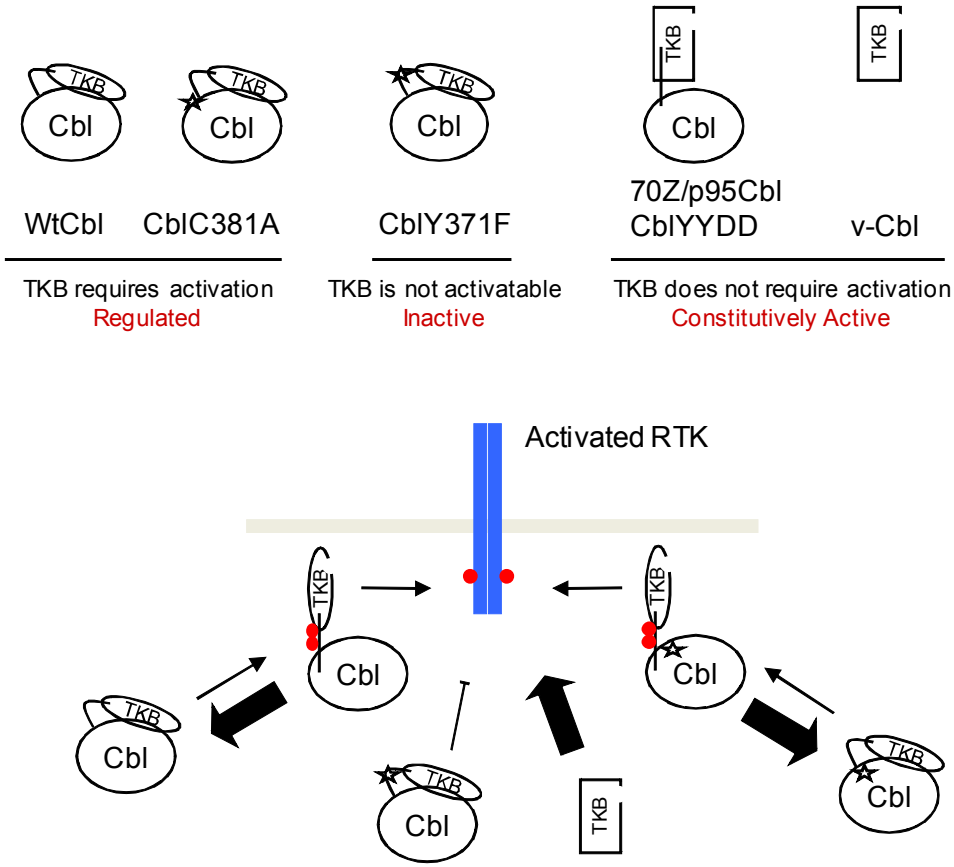


Figure 3.4 – Proposed model of Cbl-mediated transformation.

To promote cellular transformation, Cbl-mutants must lose their E3 ubiquitin-ligase activity and gain a constitutively activated TKB domain. Loss of E3 activity allows Cbl mutants to act as dominant negative proteins; however, it is the constitutive activation of the TKB domain that provides them with a competitive advantage over wtCbl in binding to Cbl-substrates and allows them to become transforming. In this schematic, Cbl proteins with unmutated Linker domains, in green, switch between tyrosine phosphorylated and dephosphorylated states, but favour the dephosphorylated state (red circles indicate tyrosine phosphorylation). Thus these proteins have a relatively lower affinity for substrates, such as activated RTKs, than transforming Cbl-mutants with unregulatable Linker domains, shown in pink. Cbl proteins with point mutations (indicated by the stars) that prevent the phosphorylation of the Linker domain, shown in blue, prevent the activation of the TKB domain and reduce the affinity of these Cbl-mutants for their substrates.

domain remains in the inactive state leaving it at a "competitive disadvantage" for binding to RTKs compared to wtCbl.

3.1.10 Cbl and Cancer

Several different transforming mutants of Cbl have been identified in mouse-derived tumours and transformed murine cell lines; including, v-Cbl, 70ZCbl, and p95Cbl (Langdon et al., 1989; Andoniou et al., 1994; Bisson et al., 2002). Recently, similar mutations in both c-Cbl and Cbl-b have been found in human acute myeloid leukemia (AML) patient samples (Caligiuri et al., 2007). Whether these Cbl mutations were causative in the AML in these patients is not yet known, however, siRNA knockdown of the mutant proteins revealed that they were important in increasing the rate of growth of AML cancer cells (Caligiuri et al., 2007).

Constitutive tyrosine phosphorylation and membrane association of Cbl, similar to that observed with the naturally occurring transforming mutants of Cbl, has also been correlated with transformation in human cancer cell lines (Kamei et al., 2000), with poor prognosis in EGFR-overexpressing human stomach cancers (Ito et al., 2004), and with leukemic cells from AML, acute lymphoblastic leukemia (ALL), and CML patients (Brizzi et al., 1998). An understanding of the mechanism of transformation of the naturally occurring transforming mutants of Cbl is necessary to understand wtCbl's role in cancer progression as well as to help determine the value of targeting the Cbl-family of proteins, both mutant and wild type, as a cancer therapy.

3.1.11 Objectives

The c-Cbl proto-oncogene is an adaptor protein and a RING finger E3 ubiquitin-ligase, and as such it acts as both a positive and negative regulator of many cellular signalling events. The mechanism by which the various naturally occurring Cbl mutants mediate cellular transformation is understood to be through the loss of its negative regulatory capacity, as all of these mutants have lost their E3 ubiquitin-ligase activity. This chapter examines an alternative hypothesis for Cbl-mediated transformation that takes into account the fact that not all E3 ubiquitin-ligase deficient Cbl mutants induce cellular transformation. I propose that mutations that alter the structure of Cbl's Linker domain provide both a gain-of-function as well as a loss-of-function, and that both are

necessary for cellular transformation. I hypothesize that mutations affecting the structural integrity of the Linker domain force Cbl to undergo a conformational change similar to that induced by the tyrosine phosphorylation of the Linker domain. However, unlike the phosphorylation of the Linker domain, the mutation-induced conformational change is irreversible and provides a gain-of-function by 'activating' or constitutively increasing the affinity of Cbl's TKB domain for its substrates. Also, unlike the phosphorylation of the Linker domain, the mutation-induced conformational change also promotes a loss-of-function, interfering with the RING finger domain's E3 ubiquitin-ligase activity and thus the negative regulation of Cbl-substrates. The combination of TKB domain activation and loss of E3 ubiquitin-ligase activity is required to generate an effective dominant negative mutant of Cbl that is able to constitutively associate with substrates but is unable to mediate their degradation.

I test this hypothesis by introducing mutations into the non-transforming, E3 ubiquitin-ligase null CblC381A mutant, which examines the involvement of the Linker domain in Cbl-mediated transformation. To encourage the tyrosine phosphorylation of the CblC381A mutant I artificially target this protein to cellular membranes by fusing it with lipid-based membrane targeting motifs. I demonstrate that these targeted Cbl mutants are constitutively tyrosine phosphorylated. This targeting also promotes Cbl-mediated transformation in a manner dependent upon Cbl's ability to associate with many of its tyrosine kinase substrates. I further validate this hypothesis by demonstrating that phosphomimetic mutations in Cbl's Linker domain also allow the CblC381A mutant to become transforming. These observations provide new insight into Cbl-mediated transformation and the role of tyrosine phosphorylation of the Linker domain in Cbl's normal function. Unexpectedly, I also show that phosphomimetic mutations in Cbl's Linker domain, on their own, are sufficient to induce cellular transformation. This observation supports a role for wtCbl in cellular transformation that is induced by unregulated tyrosine kinase activity. This implies that therapeutic strategies that target Cbl may be effective in treating cancer.

3.2 Results

To test the hypothesis that the loss of E3 ubiquitin-ligase activity and the activation of the TKB domain were both required for Cbl-mediated transformation, I used a tissue culture-based system to model Cbl-mediated transformation by overexpressing mutant Cbl proteins. The CblC381A mutant, which lacks E3 ubiquitin-ligase activity and is non-transforming, was used as the base for this system. The introduction of mutations that also cause the activation of the TKB domain would therefore allow me to specifically examine the role of TKB domain activation in Cbl-mediated transformation.

3.2.1 Artificial membrane-targeting induces the tyrosine phosphorylation of Cbl

Based on the evidence of Kassenbrock and Anderson, who demonstrated that the tyrosine phosphorylation of Cbl's Linker domain induces a conformational change that activates the TKB domain (Kassenbrock and Anderson, 2004), I was interested in generating a constitutively tyrosine phosphorylated CblC381A mutant. We and others have demonstrated that the membrane association of Cbl is correlated with its tyrosine phosphorylation (Brizzi et al., 1998; Bisson et al., 2002). Therefore, to activate the TKB domain I attempted to induce the constitutive tyrosine phosphorylation of CblC381A by fusing its amino-terminus to the membrane targeting motifs of c-Src and Csk binding protein (Cbp) (Figure 3.5). By artificially targeting Cbl mutants to cellular membranes in an unregulated manner, I hoped to force its association with many of its normal PTK substrates and induce a constitutively tyrosine phosphorylated state that would mimic the naturally occurring transforming mutants of Cbl and subsequently induce transformation.

To ascertain if the membrane targeting motifs were effectively localizing the mutant proteins to cellular membranes, I performed biochemical membrane fractionations on 3T3 mouse fibroblasts that stably expressed these Cbl proteins. Equal cell equivalents of both the cytosolic and membrane fraction were analyzed by Western blotting (Figure 3.6A), and the percentage of membrane association was determined by quantifying the Western blot band intensities and dividing the membrane value by the total of the cytosol and membrane values (Figure 3.6B). SrcCbl and CbpCbl, the control membrane-targeted mutants of wtCbl, were both more strongly associated with cellular membranes than wtCbl (Figure 3.6). Similarly, SrcCblC381A and CbpCblC381A were also more strongly

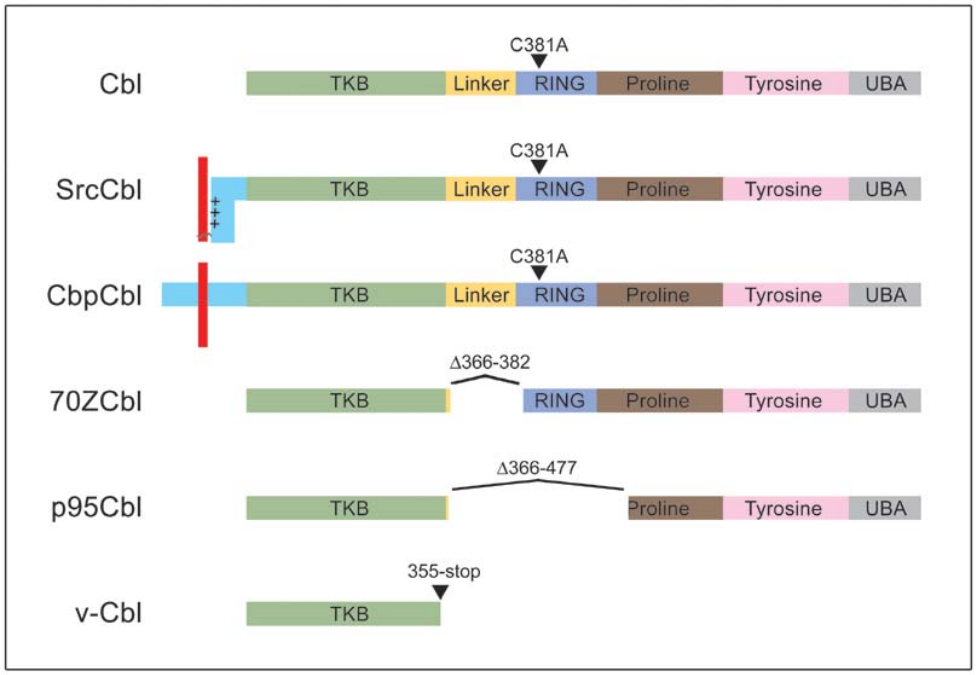


Figure 3.5 – Schematic representation of the mutant Cbl proteins used to evaluate the effect of artificial membrane-targeting on the ability of Cbl to induce cellular transformation.

Point mutations are indicated with a black arrowhead. Amino-terminal membrane targeting domains are represented in blue; with myristoylation and polybasic regions indicated for SrcCbl and a trans-membrane domain indicated for CbpCbl. Cellular membranes are represented in red.

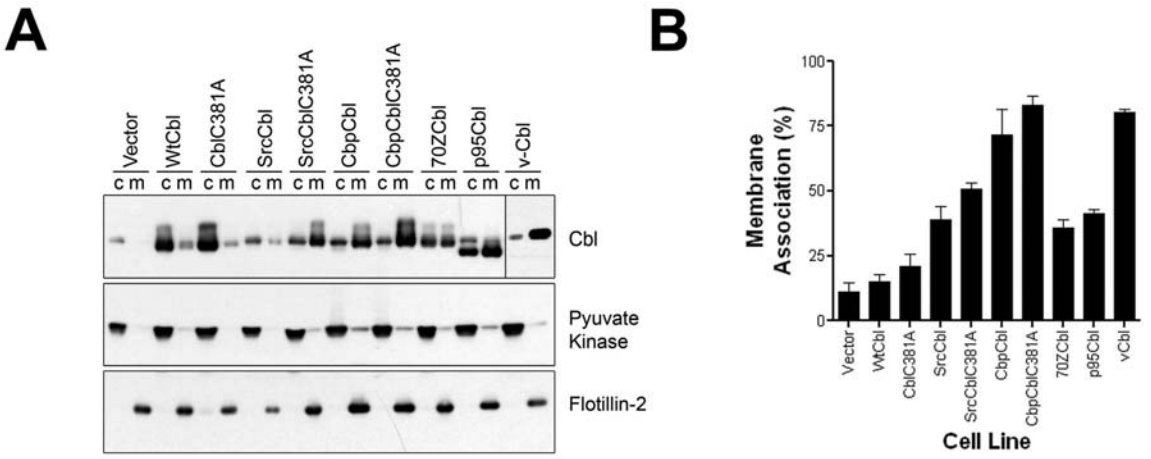


Figure 3.6 – Artificial membrane targeting motifs promote increased Cbl-membrane association.

A Western blots of biochemically isolated cytosol (c) or membrane (m) fractions from 3T3 mouse fibroblast cells stably expressing various mutant Cbl proteins. Equal cell equivalents of cytosolic and membrane fractions where run to facilitate comparison. The upper panel, probed for Cbl, indicates the mutant Cbl's relative membrane association. The mid and lower panels, probed for pyruvate kinase and flotillin2, serve as controls demonstrating the purity of the cytosolic and membrane fractions, respectively. **B** A graphical representation of Cbl membrane association derived from the quantification of Western blots of biochemically isolated cytosolic (c) and membrane (m) fractions from 3T3 mouse fibroblast cells stably expressing various mutant Cbl proteins. The percentage membrane association was calculated by dividing the amount of Cbl in the membrane fraction by the total amount of Cbl in both the cytosolic and membrane fractions. The Western blots are representative of $n \geq 3$ for two independently derived clones of each cell line. The graphical data includes data from the two independently derived clones for each cell line, with $n \geq 3$ for each clone of each cell line.

associated with cellular membranes than CblC381A (Figure 3.6). All of the membrane-targeted Cbl mutants were at least as strongly membrane associated as the naturally occurring transforming mutants of Cbl: 70ZCbl and p95Cbl (Figure 3.6). As we have previously demonstrated (Howlett and Robbins, 2002), these data indicate that membrane association motifs fused to Cbl's amino-terminus are effective at targeting Cbl to cellular membranes.

I next verified that artificial membrane-targeting of Cbl produced the intended effect of increasing its level of tyrosine phosphorylation. Cbl-mutants were immunoprecipitated from stably overexpressing 3T3 mouse fibroblasts and were examined for tyrosine phosphorylation by Western blotting (Figure 3.7). As predicted, the membrane-targeted E3 ubiquitin-ligase null mutants of Cbl were constitutively tyrosine phosphorylated (Figure 3.7). These data indicated that membrane-targeting of Cbl mutants that have lost their E3 ubiquitin-ligase activity leads to their constitutive tyrosine phosphorylation, similar to what is seen with the E3 ubiquitin-ligase null, membrane-associated, naturally occurring transforming mutants of Cbl (Andoniou et al., 1994; Bisson et al., 2002).

3.2.2 Artificial membrane-targeting of E3 ubiquitin-ligase null Cbl mutants induces cellular transformation

To determine if the tyrosine phosphorylation of CblC381A was sufficient to promote cellular transformation, I examined the ability of the membrane-targeted CblC381A mutants to support the anchorage independent growth of stably expressing 3T3 mouse fibroblasts in a soft agar colony forming assay. Targeting Cbl to cellular membranes, on its own, did not induce anchorage independent growth (Figure 3.8, SrcCbl and CbpCbl). As has been previously reported (Thien et al., 2001), the loss of E3-ligase activity alone was also not sufficient to induce cellular transformation (Figure 3.8, CblC381A). However, the combination of the loss of E3 ubiquitin-ligase activity and artificial membrane-targeting, found in both SrcCblC381A and CbpCblC381A, induced robust cellular transformation, similar to the naturally occurring transforming mutants of Cbl: 70ZCbl, p95Cbl, and v-Cbl (Figure 3.8 SrcCblC381A and CbpCblC381A). These observations indicated that the membrane targeting of E3 ubiquitin-ligase deficient

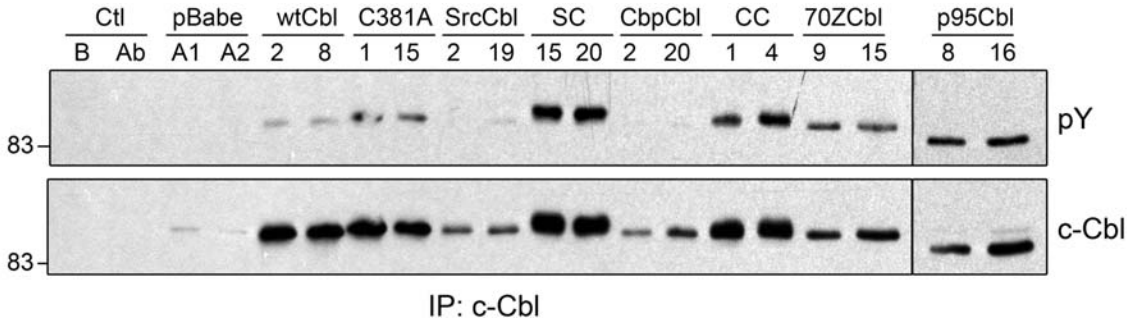


Figure 3.7 – Artificially membrane-targeted transforming mutants of Cbl are constitutively tyrosine phosphorylated.

Western blots of mutant Cbl proteins immunoprecipitated from stably expressing 3T3 mouse fibroblasts. For each Cbl mutant, immunoprecipitations were performed using two independent clones. The upper panel was probed for phosphor-tyrosine (pY), while the lower panel was reprobed for c-Cbl. SC and CC are abbreviations for SrcCblC381A and CbpCblC381A, respectively. (Representative of n=2)

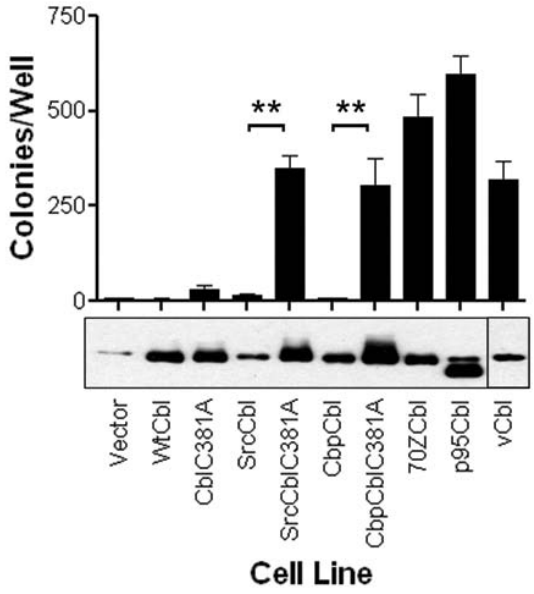


Figure 3.8 – Artificial membrane-targeting of E3 ubiquitin-ligase null Cbl mutants induces cellular transformation.

Anchorage independent growth was assayed by seeding $5x10^3$ 3T3 mouse fibroblast cells stably overexpressing various Cbl mutants in 0.35% soft agar. After 2 weeks, live colonies were stained with MTT and counted with a BioRad GelDoc 200 using the BioRad QuantityOne software. For each cell line, the graph represents $n \geq 3$ independent experiments, each with two independently derived clones. The expression level of overexpressed Cbl protein is shown by Western blotting total cell lysates from a representative clone. (** indicates $p<0.001$)

mutants of Cbl is sufficient to induce cellular transformation. They also confirmed that individually, the loss of E3 ubiquitin-ligase activity or the membrane targeting of Cbl are not sufficient to induce transformation (Thien et al., 2001; Howlett and Robbins, 2002).

3.2.3 An artificial, membrane-targeting motif is not sufficient to induce the tyrosine phosphorylation of Cbl in the absence of a functional TKB domain

I was next interested in determining whether the tyrosine phosphorylation of the membrane-targeted CblC381A mutants was due to specific TKB domain-mediated interactions with PTKs at cellular membranes or whether the tyrosine phosphorylation was due to less specific interactions facilitated by the forced membrane localization of the Cbl proteins. To test the contribution of the artificial membrane-targeting motif in the tyrosine phosphorylation status of SrcCblC381A I introduced a mutation into the TKB domain, G306E, which has previously been shown to decrease the ability of Cbl to interact with TKB domain binding partners in a phosphotyrosine dependent manner (Bonita et al., 1997) (Figure 3.9). More importantly this mutation has been shown to abrogate the ability of Cbl to become tyrosine phosphorylated by these binding partners (Miyake et al., 1999) and therefore this mutation would eliminate the contribution of the TKB domain to Cbl phosphorylation in the artificially membrane-targeted Cbl mutants.

Biochemical membrane fractionations of 3T3 mouse fibroblasts stably expressing G306E mutants of Cbl demonstrated that in the presence of an artificial membrane anchor, a functional TKB domain is not required to promote the membrane association of Cbl (Figures 3.10). However, 70ZCbl, which normally localizes to cellular membranes through TKB domain-mediated interactions with RTKs (Bonita et al., 1997), was not able to effectively localize to cellular membranes in the presence of the G306E mutation (Figure 3.10, 70ZCblG306E). These results demonstrated that the membrane-targeting motifs fused to Cbl's amino-terminus are sufficient to localize Cbl to cellular membranes in the absence of a functional TKB.

To elucidate the role of the artificial membrane-targeting motif with respect to Cbl tyrosine phosphorylation, I next examined the tyrosine phosphorylation status of the G306E Cbl mutants. Cbl was immunoprecipitated from stably expressing 3T3 mouse fibroblast cells and the tyrosine phosphorylation status was determined by Western

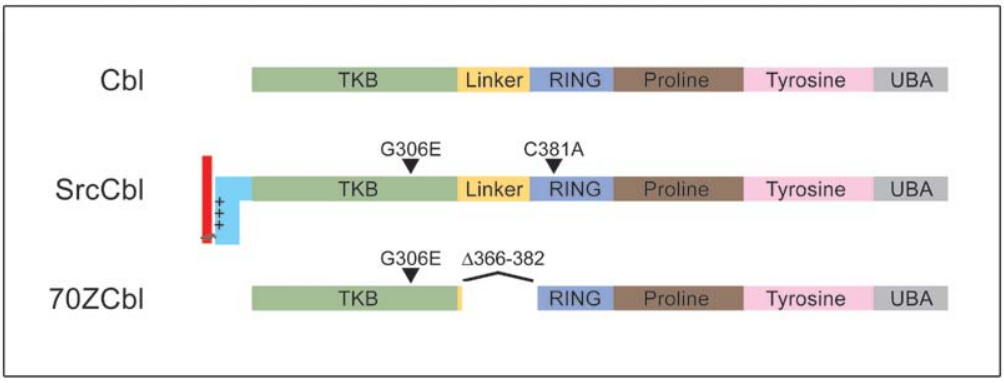


Figure 3.9 – Schematic representation of Cbl mutants used to evaluate the requirement for a functional TKB domain in Cbl-mediated transformation.

Point mutations are indicated with a black arrowhead. The amino-terminal membrane-targeting domain of SrcCbl is represented in blue and cellular membranes are represented in red.

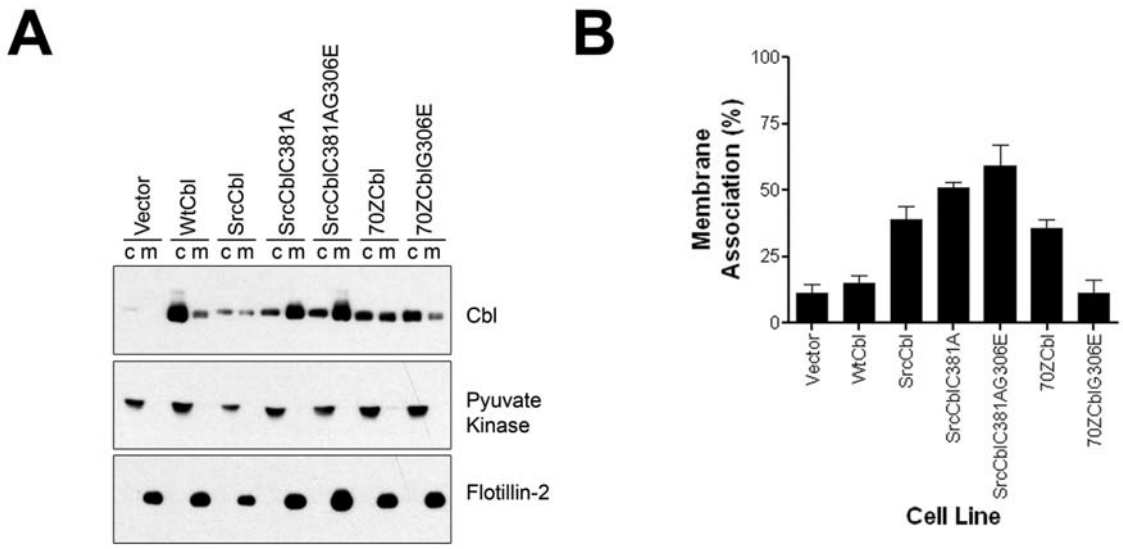


Figure 3.10 – Artificial membrane-targeting motifs promote Cbl membrane association in the absence of a functional TKB domain.

A Western blots of biochemically isolated cytosol (c) or membrane (m) fractions from 3T3 mouse fibroblast cells stably expressing various mutant Cbl proteins. Equal cell equivalents of cytosolic and membrane fractions were run to facilitate comparison. The upper panel, probed for Cbl, indicates the mutant Cbl's relative membrane association. The mid and lower panels, probed for pyruvate kinase and flotillin2, serve as controls demonstrating the purity of the cytosolic and membrane fractions, respectively. **B** A graphical representation of Cbl membrane association derived from the quantification of Western blots of biochemically isolated cytosolic (c) and membrane (m) fractions from 3T3 mouse fibroblast cells stably expressing various mutant Cbl proteins. The percentage membrane association was calculated by dividing the amount of Cbl in the membrane fraction by the total amount of Cbl in both the cytosolic and membrane fractions. The Western blots are representative of n>3 for two independently derived clones of each cell line. The graphical data includes data from the two independently derived clones for each cell line, with n>3 for each clone of each cell line.

blotting (Figure 3.11). As we had previously shown, SrcCblC381A was robustly tyrosine phosphorylated compared to wtCbl, CblC381A, and SrcCbl, indicating that forced membrane association of Cbl induced the accumulation of tyrosine phosphorylation only in the absence of E3 ubiquitin-ligase activity (Figure 3.11). The lack of detectable tyrosine phosphorylation of the G306E mutants indicated that this phosphorylation was dependent upon the TKB domain, similar to the tyrosine phosphorylation of 70ZCbl (Figure 3.11, SrcCblC381AG306E and 70ZCblG306E). The negative correlation between the presence of the G306E mutation and Cbl tyrosine phosphorylation also indicated that Cbl tyrosine phosphorylation was mediated by protein-protein interactions through the TKB domain and that membrane localization, on its own, was not sufficient to promote this event. These data suggested that membrane-targeting, *via* artificial membrane-targeting motifs and *via* the TKB domain, facilitate Cbl tyrosine phosphorylation, by localizing Cbl to a cellular compartment in which it has a higher probability of encountering activated PTKs.

3.2.4 Membrane-targeted, E3 ubiquitin-ligase null mutants of Cbl require a functional TKB domain to mediate cellular transformation

A functional TKB domain has been shown to be necessary, not only for the tyrosine phosphorylation of Cbl (Miyake et al., 1999), but also for the ability of Cbl mutants to induce cellular transformation (Bonita et al., 1997). I next wanted to confirm that the artificially membrane-targeted, E3 ubiquitin-ligase null mutants of Cbl were mediating cellular transformation in a similar dominant negative manner as the naturally occurring transforming mutants of Cbl. In order to examine this, I tested the ability of 3T3 mouse fibroblasts, stably expressing G306E mutants of Cbl, to support anchorage independent growth in a soft agar colony forming assay. In the absence of a functional TKB domain, the membrane-targeted C381A mutant of Cbl, similar to 70ZCblG306E, was unable to mediate cellular transformation (Figure 3.12, SrcCblC381AG306E and 70ZCblG306E). This experiment demonstrated that a functional TKB domain is essential for Cbl-mediated transformation even in the presence of an alternative membrane-targeting motif. This experiment also indicated that the membrane-targeted CblC381A mutant proteins are mediating cellular transformation

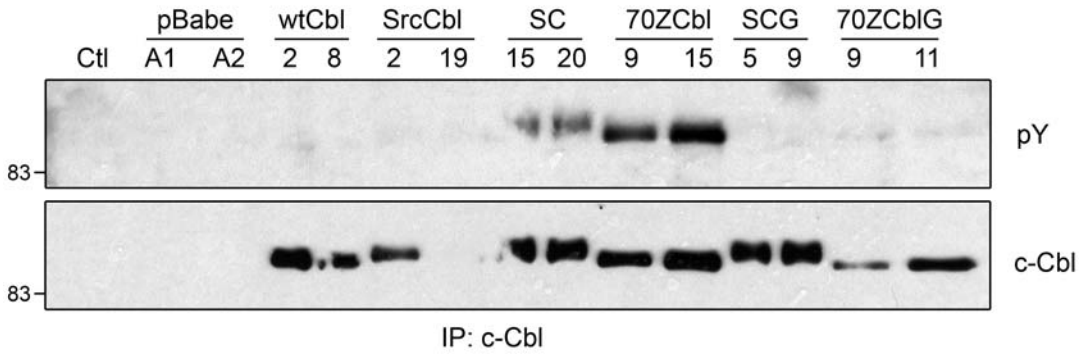


Figure 3.11 – Artificially membrane-targeted transforming mutants of Cbl are constitutively tyrosine phosphorylated in the presence of a functional TKB domain.
Western blots of mutant Cbl proteins immunoprecipitated from stably expressing 3T3 mouse fibroblasts. Immunoprecipitations were performed using two independent clones from fibroblasts expressing empty vector (pBabe), wtCbl, SrcCbl, SrcCblC381A (SC), 70ZCbl, SrcCblC381AG306E (SCG), and 70ZCblG306E (70ZCblG). The upper panel was probed for phosphor-tyrosine (pY), while the lower panel was reprobed for c-Cbl. (Representative of n=2)

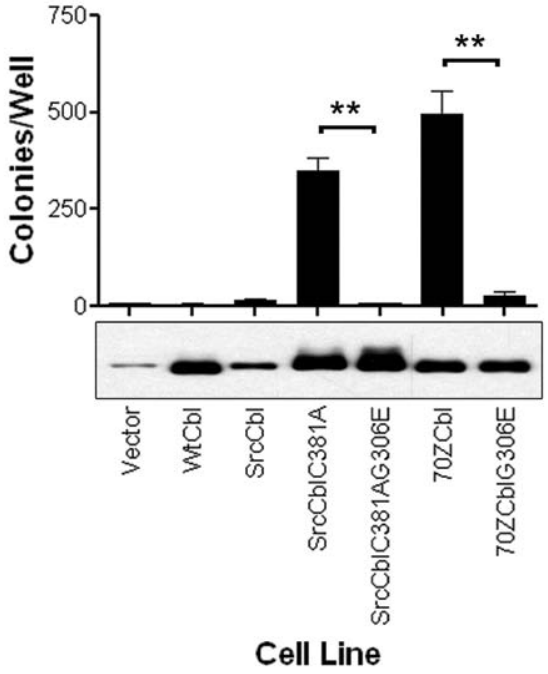


Figure 3.12 – Loss of TKB function abrogates the ability of artificially membrane targeted E3-ligase null Cbl mutants to induce cellular transformation.

Anchorage independent growth was assayed by seeding $5x10^3$ mouse 3T3 fibroblasts stably overexpressing various Cbl mutants in 0.35% soft agar. After 2 weeks, live colonies were stained with MTT and counted with a BioRad GelDoc 200 using the BioRad QuantityOne software. For each cell line, the graph represents $n \geq 3$ independent experiments, each with two independently derived clones. The expression level of overexpressed Cbl protein is shown by Western blotting total cell lysates from a representative clone for Cbl. (** indicates $p<0.001$)

through a dominant negative mechanism, similar to the naturally occurring transforming mutants of Cbl.

3.2.5 Phosphomimetic mutations in the Linker domain increase the membrane association of Cbl

The naturally occurring transforming mutants of Cbl all contain mutations in the Linker domain. I hypothesized that these mutations induce an activating conformational change in the TKB domain, similar to the conformational change induced by the tyrosine phosphorylation of the Linker domain. This phosphorylation-dependent conformational change has already been shown to increase the affinity of the TKB domain for its substrates (Kassenbrock and Anderson, 2004). Therefore, I hypothesized that the Linker domain-induced activation of the TKB domain is required for Cbl mutants to effectively mediate cellular transformation as dominant negative proteins. I have already demonstrated that the artificial membrane-targeting of CblC381A induced its tyrosine phosphorylation, which correlated with cellular transformation (Figures 3.11 and 3.12). However, as Cbl has multiple potential sites of tyrosine phosphorylation, these experiments were unable to determine whether the Linker domain tyrosine residues were being phosphorylated and therefore the role of the Linker domain in Cbl-dependent cellular transformation remained unclear.

To determine whether tyrosine phosphorylation of the CblC381A Linker domain was sufficient to permit the TKB domain-dependent dominant negative model of transformation, I introduced phosphomimetic aspartic acid residues into Cbl's Linker domain to replace the conserved tyrosine residues Y368 and Y371 (Figure 3.13, CblYYDD mutants). These phosphomimetic mutations are known to induce the activation of the TKB domain as they imitate the tyrosine phosphorylation of Cbl's Linker domain (Kassenbrock and Anderson, 2004).

To evaluate the effects of the phosphomimetic mutations, I first examined their ability to induce membrane localization (Figure 3.14), as these mutations should promote the association of Cbl with membrane-localized TKB domain substrates. Biochemical membrane fractionation of 3T3 mouse fibroblasts, stably expressing the CblYYDD mutants, demonstrated that the presence of these mutations appear to reproducibly

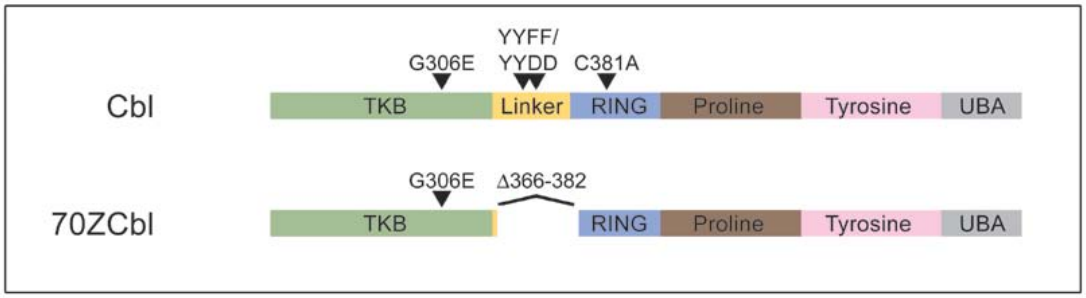


Figure 3.13 – Schematic representation of Cbl mutants used to evaluate the requirement for Linker-induced conformational change on Cbl-mediated transformation.

Point mutations are indicated with a black arrowhead.

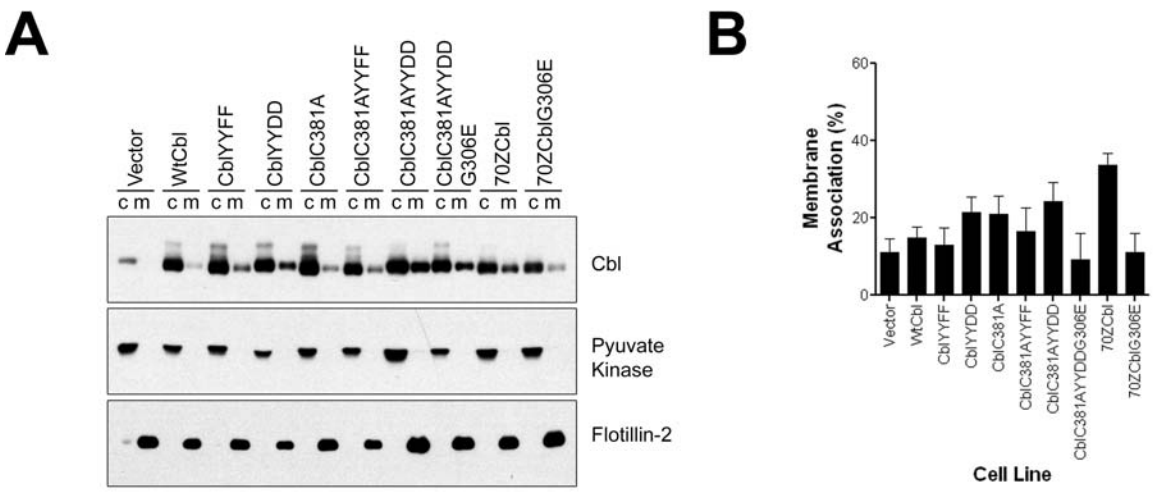


Figure 3.14 – Phosphomimetic mutations in the Linker domain alter Cbl-membrane association.

A Western blots of biochemically isolated cytosol (c) or membrane (m) fractions from 3T3 mouse fibroblast cells stably expressing various mutant Cbl proteins. Equal cell equivalents of cytosolic and membrane fractions where run to facilitate comparison. The upper panel, probed for Cbl, indicates the mutant Cbl's relative membrane association. The mid and lower panels, probed for pyruvate kinase and flotillin2, serve as controls demonstrating the purity of the cytosolic and membrane fractions, respectively. **B** A graphical representation of Cbl membrane association derived from the quantification of Western blots of biochemically isolated cytosolic (c) and membrane (m) fractions from 3T3 mouse fibroblast cells stably expressing various mutant Cbl proteins. The percentage membrane association was calculated by dividing the amount of Cbl in the membrane fraction by the total amount of Cbl in both the cytosolic and membrane fractions. The Western blots are representative of n>3 for two independently derived clones of each cell line. The graphical data includes data from the two independently derived clones for each cell line, with n>3 for each clone of each cell line.

enhance the ability of Cbl to associate with cellular membranes (Figure 3.14, wtCbl vs CblYYDD and CblC381A vs CblC381AYYDD). Accordingly, the CblYYFF mutants did not associate with cellular membranes as these mutants are unable to undergo tyrosine phosphorylation in their Linker domains and therefore are unable to undergo the Linker domain-induced conformational change that activates the TKB domain. While the differences in membrane association between these Cbl mutants and their unmodified Linker domain counterparts were not statistically significant (Figure 3.14), the same trend was apparent whether these phosphomimetic Linker domain mutations were introduced into wtCbl or into CblC381A (Figure 3.14). These data were consistent with the *in vitro* results of Kassenbrock and Anderson, who demonstrated that the introduction of phosphomimetic mutations in the Linker domain of Cbl increased its ability to associate with membrane-associated RTKs (Kassenbrock and Anderson, 2004).

3.2.6 Phosphomimetic mutations in the Linker domain induce Cbl-mediated transformation

Since I observed a trend towards increased membrane association induced by the phosphomimetic mutations in Cbl's Linker domain, we were interested in determining if these mutations were sufficient to promote cellular transformation when coupled with the loss of E3 ubiquitin-ligase activity. This would verify the requirement for the Linker domain-induced activation of the TKB domain to give the E3 ubiquitin-ligase null Cbl a competitive advantage for substrate binding and thereby verify my modified, dominant negative model of Cbl-mediated cellular transformation. Soft agar colony forming assays indicated that CblC381AYYDD induced a similar degree of transformation as the highly transforming 70ZCbl mutant (Figure 3.15). This result supported our hypothesis that mutations in Cbl's Linker domain, which force an activating conformational change in Cbl's TKB domain, promote cellular transformation. CblC381AYYFF, which contains Linker domain mutations that prevent the activation of the TKB domain, was unable to transform cells (Figure 3.15). This further supported our hypothesis in a reciprocal manner by demonstrating that Linker domain mutations that prevent the activation of the TKB domain are not transforming. Confirming that CblC381AYYDD and 70ZCbl were mediating cellular transformation through a similar dominant negative mechanism,

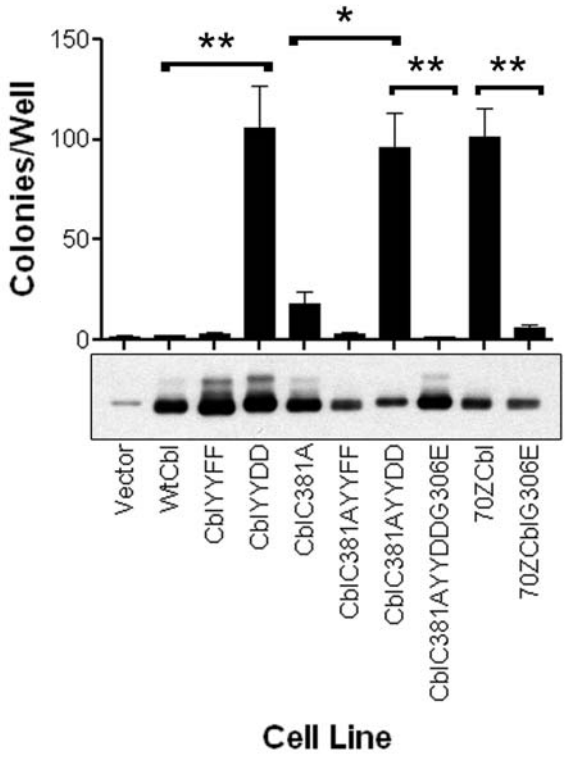


Figure 3.15 – Phosphomimetic mutations in the Linker domain induce cellular transformation.

Anchorage independent growth was assayed by seeding $5x10^3$ mouse 3T3 fibroblasts stably overexpressing various Cbl mutants in 0.35% soft agar. After 2 weeks, live colonies were stained with MTT and counted with a BioRad GelDoc 200 using the BioRad QuantityOne software. For each cell line, the graph represents at least 3 repetitions, each with two independently derived clones. The expression level of overexpressed Cbl protein is shown by Western blotting total cell lysates from a representative clone for Cbl. (* indicates $p<0.01$, and ** indicates $p<0.001$)

the loss TKB domain function, mediated by the introduction of the G306E mutation, was sufficient to abrogate cellular transformation in both of these Cbl mutants (Figure 3.15, CblC381AYYDDG306E and 70ZCblG306E).

Unexpectedly, the control CblYYDD mutant, which still possesses E3 ubiquitin-ligase activity *in vitro* (Kassenbrock and Anderson, 2004), induced a comparable level of cellular transformation as 70ZCbl (Figure 3.15, CblYYDD and 70ZCbl). These data suggested that the constitutive tyrosine phosphorylation of Cbl's Linker domain, on its own, is sufficient to induce cellular transformation.

3.3 Discussion

Dominant negative proteins, by definition, dominantly affect the normal cellular phenotype by interfering with the function of the normal gene product in the same cell (Alberts, 2002). Considerable evidence indicates that transforming Cbl mutants mediate cellular transformation through a dominant negative mechanism: transforming Cbl mutants interfere with wtCbl-mediated ubiquitination of substrate signalling molecules, leading to prolonged signalling from these proteins and resulting in cellular transformation (Swaminathan and Tsygankov, 2006). This mechanism implies that all Cbl mutants that have lost their E3 ubiquitin-ligase activity should behave as dominant negative proteins; however, loss-of-function mutations that specifically abrogate E3 ubiquitin-ligase activity, such as the CblC381A mutation, are not able to mediate robust cellular transformation. To explain this discrepancy, I hypothesized that a transforming Cbl mutation would have to induce a loss-of-function with respect to E3 ubiquitin-ligase activity and also induce a gain-of-function by increasing the affinity of the TKB domain for membrane associated Cbl-substrates. This type of mutation would give these Cbl mutants a competitive advantage over wtCbl in their ability to associate with TKB domain substrates, thus increasing their success as dominant negative proteins.

To test this hypothesis, I developed a model of Cbl-mediated transformation in which distinct mutations were used to induce this loss- or gain-of-function. The loss-of-function was provided by the C381A mutation, which has been shown to abrogate E3 ubiquitin-ligase activity (Joazeiro et al., 1999). Two separate approaches were used to generate the gain-of-function in the TKB domain. These approaches were based the

recent demonstration that the tyrosine phosphorylation of Cbl's Linker domain promotes the activation of the TKB domain by inducing a conformational change (Kassenbrock and Anderson, 2004).

The first approach involved artificially targeting CblC381A to cellular membranes by fusing the membrane association motifs of either Src or Cbp to Cbl's amino-terminus. This approach was based on the observation that the membrane association of both wtCbl and the naturally occurring transforming Cbl mutants is associated with Cbl tyrosine phosphorylation (Brizzi et al., 1998; Bisson et al., 2002; Bisson, 2004; Kassenbrock and Anderson, 2004). I demonstrated that artificial membrane targeting of CblC381A does induce its tyrosine phosphorylation and that this correlates with its ability to transform cells (Figures 3.7 and 3.8).

The second approach was used to further confirm the link between the tyrosine phosphorylation of Cbl's Linker domain and the ability of CblC381A to transform cells. To directly correlate phosphorylation of the Linker domain with cellular transformation, I introduced phosphomimetic mutations into the Linker domain of CblC381A. I demonstrated that the combination of these mutations increases the membrane association of the mutant proteins, indicating that they induce a gain-of-function by increasing the TKB domain's affinity for membrane-associated Cbl binding partners (Figure 3.14). I also demonstrated that the E3 ubiquitin-ligase null, phosphomimetic Cbl mutant induces cellular transformation (Figure 3.15). This bolstered the hypothesis that phosphorylation of the Linker domain induces the activation of the TKB domain allowing for transformation in the absence of E3 ubiquitin-ligase activity. Interestingly, I also found that the phosphomimetic mutations, in the presence of a functional RING finger domain, were transforming. This observation implicates constitutively tyrosine phosphorylated wtCbl in cellular transformation.

Considering that the only common feature of all of the known transforming mutants of Cbl is the mutation or deletion of the Linker domain, these data support a model of Cbl transformation in which these Linker domain mutations abrogate E3 ubiquitin-ligase activity and force an activating conformational change in Cbl's TKB domain. Unlike the previous dominant negative model, this new model for Cbl-mediated

transformation can explain the behaviour of all of the E3 ubiquitin-ligase deficient mutants of Cbl and also adds to the understanding of the mechanism of action of wtCbl.

3.3.1 CblC381A

The loss of E3 ubiquitin-ligase activity should be sufficient for Cbl to behave as a dominant negative protein, by competing with wtCbl for binding to substrates and thus preventing the appropriate ubiquitination and downregulation of those substrates. However, CblC381A is not robustly transforming when overexpressed in murine 3T3 fibroblast cells (Figure 3.8) (Thien et al., 2001). My proposed model for Cbl-mediated transformation explains the inability of the C381A E3 ubiquitin-ligase null Cbl mutant to mediate cellular transformation. This Cbl mutant, unlike the naturally occurring transforming mutants of Cbl, does not possess Linker domain mutations and therefore its TKB domain activity is regulated in a similar manner to endogenous wtCbl. This is supported by the observation that CblC381A was no more membrane associated or tyrosine phosphorylated than overexpressed wtCbl (Figures 3.6 and 3.7). Under these circumstances, neither protein has an advantage for binding to tyrosine phosphorylated Cbl-substrates, allowing these substrates to be effectively ubiquitinated. The low level of cellular transformation observed in soft agar colony forming assays for CblC381A-expressing 3T3 mouse fibroblasts is likely due to the high ratio of overexpressed CblC381A to endogenous Cbl in these cells (Figure 3.8 and 3.15: compare Cbl levels between pBabe and CblC381A). This ratio, in support of CblC381A, would favour the binding of CblC381A to TKB domain substrates over wtCbl and allow it to function as a weak dominant negative protein. This observation also indicates that very little Cbl-mediated E3 ubiquitin-ligase activity is required to maintain cellular homeostasis and supports the requirement for transforming mutants of Cbl to have increased TKB domain activity to effectively function as dominant negative proteins.

3.3.2 CblY371F

According to my model for Cbl-mediated transformation, despite the loss of E3 ubiquitin-ligase activity, CblY371F is not transforming due to the inability of this mutant to activate its TKB domain. In this respect, this mutant cannot act in a dominant negative manner as it has a reduced ability to bind to tyrosine phosphorylated substrates. This

reduced ability cannot be increased through tyrosine phosphorylation, making it less competitive than endogenous wtCbl for its normal substrates (Kassenbrock and Anderson, 2004). Consequently, I observed that the CblC381AYYFF and CblYYFF mutants were less strongly associated with cellular membranes than CblC381A and wtCbl, respectively (Figure 3.14). The residual membrane association of these mutants is likely mediated by phosphotyrosine-independent interactions with adaptor proteins such as Grb2 and SLAP, which have been shown to be sufficient to localize Cbl to cellular membranes in the absence of a functional TKB (Wang et al., 1999; Waterman et al., 2002; Penengo et al., 2003; Grovdal et al., 2004; Sun et al., 2007; Swaminathan et al., 2007).

3.3.3 Naturally occurring transforming mutants of Cbl

Naturally occurring transforming mutants of Cbl conform to our criteria for transforming mutants of Cbl through their loss of the Linker and RING finger domains. Our model for Cbl-mediated transformation requires the loss of E3 ubiquitin-ligase activity and unregulated activation of the TKB domain. The loss of E3 ubiquitin-ligase activity in the naturally occurring, transforming Cbl mutants is the result of the partial or complete deletion of Cbl's RING finger domain in these mutants. The mutation of the critical RING finger domain C381 residue, which is deleted in all three naturally occurring Cbl mutants, has been shown to abrogate E3 ubiquitin-ligase activity by interfering with Cbl's ability to associate with E2 ubiquitin-conjugating enzymes (Joazeiro et al., 1999).

I believe that the deletion of the Linker domain in v-Cbl, 70ZCbl, and p95Cbl promotes a similar activating conformational change as is induced by the tyrosine phosphorylation of the Linker domain (Kassenbrock and Anderson, 2004). This is supported by the observation that all of the naturally occurring transforming Cbl-mutants have an increased affinity for cellular membranes, similar to the Linker domain phosphomimetic mutants of Cbl (Figure 3.14). It is also supported by our observation that the phosphomimetic Linker domain mutations induce cellular transformation to a similar degree as 70ZCbl (Figure 3.15).

A comparison between the naturally occurring transforming mutants of Cbl, despite their similar mechanism of action, reveals differences in their ability to transform cells. The smallest of these Cbl mutants, v-Cbl, is more strongly membrane associated than 70ZCbl or p95Cbl (Figure 3.5). This observation may be demonstrative that the carboxy-terminal region of Cbl negatively regulates its membrane association. Compared with v-Cbl, the carboxy-terminal regions of p95Cbl and 70ZCbl may interfere with their ability to associate with cellular membranes through interactions with Sprouty proteins, through CIN85/endophilin-dependent negative regulation of RTKs, and through dimerization with and subsequent ubiquitination by endogenous wtCbl (Swaminathan and Tsygankov, 2006). However, loss of Cbl's carboxy-terminal region also appears to have a detrimental effect on v-Cbl's ability to induce cellular transformation: 70ZCbl and p95Cbl are between 30 to 50% more transforming in a soft agar colony forming assay (Figure 3.7). This implies that the protein-protein interactions mediated by carboxy-terminal regions of the transforming mutants of Cbl play an important role in augmenting their transforming effects, presumably by facilitating the recruitment of signalling effector proteins to activated RTKs and promoting a more diverse signalling response (Bonita et al., 1997). This diverse response may not only reside in the ability of 70ZCbl and p95Cbl to recruit a wider variety of signalling effectors, due to their carboxy-terminal protein-protein interaction motifs, but may also be the result of the ability of these proteins to interact with CIN85/endophilin, promoting the endocytosis of associated RTKs independently from E3 ubiquitin-ligase activity (Swaminathan and Tsygankov, 2006). Since RTK signalling from the endosomal compartment has been shown to be important in the mitogenic responses to RTK activation (Maudsley et al., 2000; Shen et al., 2001), this may explain the increased potency of transformation mediated by 70ZCbl and p95Cbl compared to v-Cbl.

3.3.4 SrcCblC381A and CbpCblC381A

Similar to the naturally occurring transforming mutants of Cbl, SrcCblC381A and CbpCblC381A do not possess E3 ubiquitin-ligase activity and have constitutively activated TKB domains. However, unlike the naturally occurring transforming mutants of Cbl, the loss of E3 ubiquitin-ligase activity and the activation of the TKB domain are

induced by two separate mutations in these artificially generated transforming Cbl mutants: the C381A mutation is responsible for the loss of E3 ubiquitin-ligase activity (Joazeiro et al., 1999; Thien et al., 2001) whereas the artificial membrane targeting, which induces constitutive tyrosine phosphorylation of the mutant Cbl proteins (Figure 3.6), is responsible for the activation of the TKB domain (Kassenbrock and Anderson, 2004). Despite the fact that the Linker domain in these membrane-targeted Cbl mutants can still be regulated by the addition or removal of tyrosine phosphorylation, constitutive tyrosine phosphorylation is likely induced through a self reinforcing positive regulatory loop: the targeting of a dominant negative Cbl protein to the plasma membrane reduces the downregulation of activated RTKs and associated signalling effector proteins, thereby increasing the tyrosine phosphorylation of Cbl, which increases its effectiveness as a dominant negative protein by enhancing its TKB domain-mediated affinity for substrate proteins. Thus, while SrcCblC381A and CbpCblC381A require two separate mutations to induce cellular transformation, the C318A mutation to abrogate E3 ubiquitin-ligase activity and the membrane targeting motifs to promote TKB domain activation, they possess comparable characteristics and behave in a similar manner as the naturally occurring transforming mutants of Cbl.

3.3.5 CblC381AYYDD

To verify the hypothesis that tyrosine phosphorylation of the Linker domain induced an activating conformational change in the TKB domain that mimicked the conformation of the naturally occurring transforming mutants of Cbl, I introduced phosphomimetic point mutations into the Linker domain of CblC381A, generating CblC381AYYDD. This mutant most closely mimics the naturally occurring transforming mutants of Cbl as it has lost its E3 ubiquitin-ligase activity and possesses phosphomimetic point mutations, which constitutively activate the TKB domain (Kassenbrock and Anderson, 2004). Accordingly, I found an increase in the association of this CblC381AYYDD mutant with cellular membranes compared with CblC381A (Figure 3.14). The increased membrane association, as predicted by my model, also correlated with an increase in the ability of Cbl to transform cells: CblC381AYYDD was as transforming as 70ZCbl (Figure 3.15). Similar to 70ZCbl, the introduction of the

G306E mutation into the TKB domain of CblC381AYYDD also decreased its ability to associate with cellular membranes (Figure 3.14) and abrogated its ability to transform cells (Figure 3.15). This similarity and the similarities in membrane association and transforming ability between CblC381AYYDD and the naturally occurring transforming mutants of Cbl, suggest that these mutant Cbl proteins mediate cellular transformation through a similar mechanism. Therefore, CblC381AYYDD demonstrates that Cbl-mediated cellular transformation is induced by a combination of the loss of E3 ubiquitin-ligase activity and the activation of the TKB domain.

3.3.6 CblΔY368 and CblΔY371

Although CblΔY368 and CblΔY371, are simply point mutations, these artificially generated Cbl mutants are more potent transforming agents than 70ZCbl (Thien et al., 2001). According to my model for Cbl-mediated transformation, perturbation of the Linker domain with either of these point deletions must be sufficient to activate the TKB domain and abrogate E3 ubquitin-ligase activity. As previously mentioned, I believe that perturbation of the Linker domain structure is sufficient to force an activating conformational change in the TKB domain. However, the mechanism by which these point mutants abrogate E3 ubiquitin-ligase activity is less clear. Complicating this understanding is the fact that while CblΔY368 has been shown to have no E3 ubiquitin-ligase activity *in vivo* (Thien et al., 2001), this mutant maintains the ability to associate with E2 ubiquitin-conjugating enzymes and to induce ubiquitination *in vitro* (Joazeiro et al., 1999).

These discrepancies can be explained through a more detailed understanding of the ubiquitination process. Cbl is recruited from the cytosol to the plasma membrane through its constitutive association with adapter proteins, such as, Grb2 and SLAP (Waterman et al., 2002; Sun et al., 2007; Swaminathan et al., 2007). At the plasma membrane, PTKs mediate the tyrosine phosphorylation of Cbl's Linker domain leading to conformational changes that allows the TKB domain to interact with tyrosine phosphorylated protein substrates. This conformational change also increases Cbl's E3 ubiquitin-ligase activity (Kassenbrock and Anderson, 2004). Protein-protein interactions between E2 ubiquitin-conjugating enzyme and Cbl's RING finger and Linker domains

allosterically activate the E2 enzyme, promoting its ability to transfer ubiquitin to substrate proteins (Ozkan et al., 2005). Once it has mediated the transfer of a ubiquitin molecule to a target protein, the RING finger must release the spent E2 enzyme and associate with a new ubiquitin-loaded E2 (Eletr et al., 2005). Concurrent with the ubiquitination process, deubiquitination enzymes proteolytically remove ubiquitin molecules from substrate proteins: the balance between active ubiquitination and deubiquitination thus determines the ubiquitination status of target proteins (Wing, 2003).

Three of these mechanistic features are likely involved in the contrasting *in vitro* and *in vivo* observations concerning CblΔY368's E3 ubiquitin-ligase activity: 1) The ability of CblΔY368 to release spent E2 ubiquitin-conjugating enzymes, 2) the ability of CblΔY368 to interact with new ubiquitin-loaded E2 enzymes, and 3) the ability of CblΔY368 to efficiently mediate substrate ubiquitination in the presence of deubiquitinating enzymes. Deletion of Y368 may alter the conformation of the Linker domain in such a manner that it cannot properly release bound E2 enzymes. In this instance, *in vitro* ubiquitination would still be observed; however, *in vivo* ubiquitination would be compromised as each mutant Cbl protein would only be able to mediate the transfer of one ubiquitin molecule before effectively losing its E3 ubiquitin-ligase activity due to its inability to recruit new ubiquitin-loaded E2 enzymes.

Alternatively, if CblΔY368 was able to bind and release E2 enzymes normally, this protein would still need to move to a cellular compartment in which the ratio of ubiquitin associated E2 enzymes to ubiquitin dissociated E2 enzymes favours the former. We believe that the ΔY368 mutation constitutively activates the TKB domain; under these circumstances this protein would be strongly biased towards remaining bound to RTKs and remain localized near the plasma membrane. As many other ubiquitin ligases are present near the plasma membrane this cellular compartment likely favours higher concentrations of ubiquitin-dissociated E2 enzymes. Therefore, it would be expected that after a Cbl protein has mediated the transfer of a ubiquitin molecule from a ubiquitin-associated E2 enzyme, it must relocalize to another cellular compartment to 'reload' with a new ubiquitin-associated E2 enzyme. In the presence of the TKB domain activating mutations, this would be unlikely to occur, limiting CblΔY368's ability to act as a

functional E3 ubiquitin-ligase. However, *in vitro*, in the presence of excess ubiquitin-associated E2 enzyme, CblΔY368 would be able to effectively ubiquitinate substrate proteins.

Finally, mutations of several residues involved in the interaction between the E2 enzymes and RING finger proteins have been shown to interfere with the ability of E2 enzymes to mediate ubiquitination without affecting their ability to interact with RING fingers (Ozkan et al., 2005). Thus mutation of Cbl's Linker domain, while not affecting the ability to interact with E2 ubiquitin-conjugating enzymes (Joazeiro et al., 1999), may still be able to affect the rate of transfer of ubiquitin from the E2 enzymes to substrate proteins. *In vitro*, this would not significantly affect assays that use excess amounts of ubiquitinating enzymes from detecting the presence of ubiquitinated substrates. However, *in vivo*, in an environment containing limited amounts of ubiquitinating enzymes and active deubiquitinating enzymes, alterations in the rate of transfer of ubiquitin to substrate proteins may shift the ubiquitination equilibrium of these substrates towards deubiquitination, making such Cbl mutants appear to have no E3 ubiquitin-ligase activity.

Of the three mechanistic defects outlined, I believe that the conflict between the *in vitro* and *in vivo* E3 ubiqutin-ligase activity of CblΔY368 is most likely explained by its inability to relocate to cellular compartments with higher concentrations of ubiquitin-associated E2 ubiquitin-conjugating enzymes. This is supported by the observations of Joazeiro and colleagues, who found that, *in vitro*, CblΔY368 associated with E2 enzymes and mediated substrate ubiquitination equivalent to wtCbl (Joazeiro et al., 1999).

3.3.7 CblYYDD

Phosphomimetic mutations in Cbl's Linker domain have been shown to increase its ability to mediate substrate ubiquitination *in vitro* (Kassenbrock and Anderson, 2004). However, similar to the confounding observations made with CblΔY368, we have determined that phosphomimetic mutations in wtCbl induce cellular transformation *in vivo* (Figure 3.15). We demonstrate that despite its reported *in vitro* E3 ubiquitin-ligase activity, the phosphomimetic Cbl mutant, CblYYDD, is able to mediate cellular transformation to a similar degree as 70ZCbl in a soft agar colony forming assay (Figure

3.15). As my model for Cbl-mediated transformation predicts the requirement for the loss of E3 ubiquitin-ligase activity as well as the activation of the TKB domain, the phosphomimetic mutations must accomplish both effects *in vivo*.

As mentioned with CblΔY368, which also demonstrates *in vitro* E3 ubiquitin-ligase activity and *in vivo* transformation capability, several differences between the *in vivo* and *in vitro* reaction conditions may explain this result. CblYYDD's ability to release spent E2 enzymes, to recruit new E2 enzymes, and to efficiently activate the transfer of ubiquitin to target substrates in the presence of deubiquitinating enzymes must all be considered. Of these variables, it is unlikely that the CblYYDD phosphomimetic mutants are affecting its ability to effectively mediate the transfer of ubiquitin to substrates because these mutations mimic the natural process of tyrosine phosphorylation that occurs within cells. In addition, *in vitro*, these phosphomimetic mutations mediate ubiquitin transfer more effectively than wtCbl (Kassenbrock and Anderson, 2004). Thus the ability to release and/or recruit E2 enzymes is more likely responsible for the contrast between the *in vitro* and *in vivo* observations. This is supported by the x-ray crystallographic study of Cbl complexed with the E2 enzyme UbcH7: amino acids CblE369 and CblE366 were shown to form salt bridges with UbcH7R5 and UbcH7R15, respectively (Zheng et al., 2000). The additional negative charge introduced by the phosphorylation, or phosphomimetic mutations, of the adjacent amino acids CblY371 and CblY368 would potentially lead to stronger interactions with the two positively charged UbcH7 arginine residues and result in an increase in the strength of association between Cbl and the E2 enzyme. This could increase Cbl's affinity for E2 enzymes and could therefore increase the apparent E3 ubiquitin-ligase activity *in vitro* where excess components are added and activity is measured over a limited time period. However, *in vivo*, phosphomimetic mutations or constitutive tyrosine phosphorylation may increase the strength of association between Cbl and associated E2 ubiquitin-conjugating enzymes such that once an E2 enzyme becomes associated with Cbl, it cannot be easily released. This would prevent Cbl from transferring more than one ubiquitin molecule to a target substrate, effectively inhibiting E3 ubiquitin-ligase activity *in vivo*. Due to the irreversible nature of the phosphomimetic mutations, CblYYDD's constitutively

activated TKB domain would therefore mediate a strong dominant negative effect by preventing other ubiquitin-loaded wtCbl-E2 complexes from transferring additional ubiquitin molecules to target substrates. The activated TKB domain would also reduce the ability of CblYYDD to move to other cellular compartments that contain higher concentrations of ubiquitin-loaded E2 enzymes, further reducing the ability of this protein to mediate ubiquitination.

3.3.8 Cbl transformation and the regulation of the TKB domain

My data indicate that the Linker domain's regulation of the activation of the TKB domain is important for Cbl-mediated transformation. This is further supported by two key observations: 1) the mutation of the Linker domain is the only common feature between all of the different natural occurring and artificially generated transforming mutants of Cbl (Figures 3.1 and 3.2) and 2) the introduction of the G306E mutation in the TKB domain, which abrogates the ability of this domain to associate with its tyrosine phosphorylated binding partners, inhibits the ability of Cbl mutants to transform cells (Bonita et al., 1997) (Figures 3.12 and 3.15).

The importance of the ability to regulate the activity of Cbl's TKB domain becomes more apparent when examining the membrane-targeted Cbl mutants SrcCbl and CbpCbl. Our observations with SrcCblC381A and CbpCblC381A indicate that the artificial membrane-targeting of Cbl induces its constitutive tyrosine phosphorylation (Figures 3.7); however, SrcCbl and CbpCbl do not appear to be tyrosine phosphorylated (Figure 3.7), nor are they transforming (Figure 3.8). SrcCbl and CbpCbl also had much lower cellular expression levels than their C381A counterparts and wtCbl (Figure 3.8), and their expression was difficult to maintain in stably expressing pools of 3T3 mouse fibroblasts (data not shown). These observations suggest that expression of the membrane-targeted Cbl proteins induced the growth inhibition of cells: overexpression of membrane-targeted Cbl reduced growth factor signalling, leading to the selection of cell clones with little or no expression of these mutant proteins. Considering the observation that tyrosine phosphorylation of Cbl *in vitro* induces E3 ubiquitin-ligase activity and auto-ubiquitination (Kassenbrock and Anderson, 2004), all of these observations concerning SrcCbl and CbpCbl can be explained by a tyrosine phosphorylation-induced

induction of E3 ubiquitin-ligase activity. Membrane-targeting of these Cbl mutants, similar to their C381A counterparts, places them in proximity to tyrosine kinases where they can be phosphorylated. Unlike their C381A counterparts, these membrane-targeted Cbl mutants have functional E3 ubiquitin-ligase activity and are thus able to mediate the ubiquitination of their substrates and of themselves. This ability to ubiquitinate negatively impacts cellular proliferation signals, leading to the selection of stably expressing clones with low expression levels of mutant membrane-targeted Cbl and leading to the selection of non-expressing populations within pools of stably transduced cells. These observations indicated that the constitutive tyrosine phosphorylation of the Linker domain must be coupled with the loss of E3 ubiquitin-ligase activity for Cbl to function as a dominant negative protein.

The question remains why these membrane-targeted Cbl mutants, if their tyrosine phosphorylation status is enhanced, do not behave in a similar manner to the phosphomimetic Cbl mutant, CblYYDD. This is likely attributable to the ability of the TKB domain of SrcCbl and CbpCbl, unlike that of CblYYDD, to be regulated by tyrosine phosphorylation and dephosphorylation. After mediating the tyrosine phosphorylation-enhanced transfer of ubiquitin from an associated E2 ubiquitin-conjugating enzyme, SrcCbl and CbpCbl can disassociate from the target substrate through dephosphorylation of the Linker domain. This ability to be regulated allows other ubiquitin-associated Cbl proteins to interact with the target substrate while the original Cbl protein recruits a new ubiquitin-conjugated E2 enzyme. In this manner, the membrane-targeted Cbl mutants do not behave as dominant negative proteins as their TKB domain-dependent affinity for substrates is regulated, allowing them to maintain their E3 ubiquitin-ligase activity.

3.3.9 New insight into the function of wild-type Cbl

While focused on determining the mechanism by which mutants of Cbl mediate cellular transformation, my data also provides additional insight into the function of wtCbl. The transforming nature of the CblYYDD mutant implies that under normal circumstances Cbl must cycle on and off of substrates at the plasma membrane to maintain its normal function. This cycling requires that Cbl's Linker domain be capable of cycling between tyrosine phosphorylation states. The cycling of Cbl to and from its

substrates presumably allows Cbl to continually recruit ubiquitin-conjugated E2 enzymes, and is required for Cbl to maintain its E3 ubiquitin-ligase activity *in vivo* (Figure 3.16).

This view of Cbl function is supported by the effective loss of Cbl's E3 ubiquitin-ligase activity when its ability to switch between tyrosine phosphorylation states is compromised. Mutations that mimic the constitutive tyrosine phosphorylation or dephosphorylation of Cbl's Linker domain effectively block its negative regulatory capabilities in cells (Figure 3.15) (Kassenbrock and Anderson, 2004). Consistent with these observations, cells in which Cbl's ability to cycle is compromised due to the failure of Grb2 to facilitate Cbl's association with activated RTKs, also demonstrate a reduction in substrate ubiquitination (Sun et al., 2007). The cycling of Cbl to and from its substrates, in a tyrosine phosphorylation dependent manner, is further supported by the inability of CblC381A to mediate transformation (Figure 3.7): only in a system where Cbl was constantly cycling could the loss of the Cbl's E3 ubiquitin-ligase activity not induce dominant negative cellular transformation.

Interestingly, several so-called negative regulators of Cbl E3 ubiquitin-ligase activity may be involved in facilitating the cycling of Cbl to and from its target substrates by impairing its protein-protein-interactions with these substrates. As a consequence, the loss of these proteins may also promote cellular transformation due to the loss of Cbl regulation, resulting in an increase in interactions between the TKB domain and its binding partners.

3.3.10 A new role for Sprouty

One such negative regulator of Cbl is the Sprouty family member Sprouty2 (Spry2): this protein has been shown to bind to Cbl's RING finger domain, blocking its ability to recruit E2 ubiquitin-conjugating enzymes (Rubin et al., 2003). In conflict with the role of Spry2 as a negative regulator of Cbl function, Spry2 is also viewed as an antagonist to RTK signalling (Guy et al., 2003; Rubin et al., 2005). Originally identified in *Drosophila melanogaster*, the loss-of-function dSpry mutant demonstrated excessive branching in the lung due to excessive FGFR mediated signalling (Hacohen et al., 1998; Casci et al., 1999). In addition, the loss of Spry2 in mammalian tissue culture has been shown to induce cellular transformation (Fong et al., 2006). Loss of Spry2 has also been

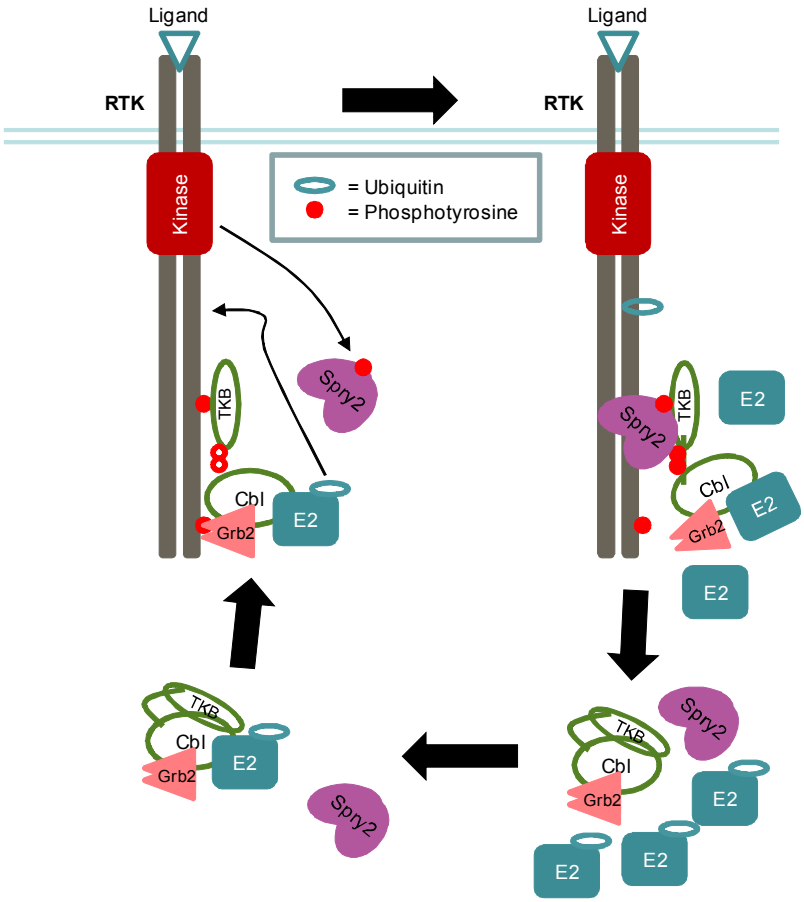


Figure 3.16 – E3 ubiquitin-ligase activity requires cycling to and from substrates. Cbl, pre-associated with a ubiquitin-loaded E2 enzyme, is recruited to activated RTKs through its association with Grb2. Cbl then becomes tyrosine phosphorylated within the Linker domain, activating its TKB domain to strongly associate with target substrates and inducing its E3 ubiquitin-ligase activity towards those substrates. Spry2 also becomes tyrosine phosphorylated, displaces previously bound tyrosine phosphorylated RTKs from Cbl's TKB domain, and thereby permits both proteins to translocate to cellular compartments where they can be dephosphorylated. Dephosphorylation of Spry2 allows it to dissociate from Cbl's TKB domain. Dephosphorylation of Cbl's Linker domain decreases its affinity for associated and spent E2 ubiquitin-conjugating enzymes and allows Cbl to associate with new ubiquitin-conjugated E2 enzymes. Subsequently, Grb2-mediated recruitment of Cbl to activated RTKs allows the cycle of Cbl-mediated ubiquitination to continue.

shown to contribute to the severity of human malignancies of the lung and liver (Fong et al., 2006; Sutterluty et al., 2007). These observations are intriguing as one would expect that the loss of a negative regulator (Spry2), of a negative regulator (Cbl), would lead to a depression of the regulated signalling processes. As the opposite is observed, it implies that this view of Spry2 function is incorrect.

By examining two well characterized molecular properties of Spry2, we believe that Spry2 plays a major role in the normal functioning of Cbl by positively regulating Cbl's ability to ubiquitinate substrate proteins. 1) When overexpressed, Spry2 has been shown to associate with Cbl through its RING finger domain, preventing Cbl from associating with E2 ubiquitin-conjugating enzymes (Rubin et al., 2003). 2) The association between Cbl and Spry2 has also been shown to promote the tyrosine phosphorylation of Spry2, which in turn allows it to bind to Cbl's TKB domain in a phosphotyrosine dependent manner (Fong et al., 2003). These two properties fit a model for Cbl regulation in which Spry2 displaces target substrates from interacting with Cbl's TKB domain, allowing Cbl to recruit new ubiquitin-conjugated E2 enzymes, propagating the ubiquitination process (Figure 3.16).

In this model, Cbl, pre-associated with a ubiquitin-loaded E2 enzyme, is recruited to activated RTKs through its association with Grb2 (Figure 3.16) (Wang et al., 1999; Waterman et al., 2002; Penengo et al., 2003; Grovdal et al., 2004; Sun et al., 2007). At the plasma membrane, Cbl becomes tyrosine phosphorylated within the Linker domain, activating its TKB domain to strongly associate with target substrates and inducing its E3 ubiquitin-ligase activity towards those substrates. Spry2 also becomes tyrosine phosphorylated and in turn binds to Cbl's activated TKB domain. Phosphorylated Spry2 displaces previously bound tyrosine phosphorylated RTKs from Cbl's TKB domain, and thereby permits both proteins to translocate to cellular compartments where they can be dephosphorylated. Dephosphorylation of Spry2 allows it to dissociate from Cbl's TKB domain. Dephosphorylation of Cbl's Linker domain decreases its affinity for associated and spent E2 ubiquitin-conjugating enzymes and allows Cbl to associate with new ubiquitin-conjugated E2 enzymes. Subsequently, Grb2-mediated recruitment of Cbl to activated RTKs allows the cycle of Cbl-mediated ubiquitination to continue (Figure

3.16). As RTK activation also induces an increase in Spry2 levels over time, this increases the efficiency of Cbl-mediated substrate ubiquitination by increasing the rate of exchange of E2 enzymes, effectively terminating RTK signalling. Increased Spry2 levels also compete with E2 enzymes for binding to Cbl's RING finger, therefore after a threshold amount of Spry2 is produced, Cbl becomes much less efficient at mediating substrate ubiquitination, resetting the cell's ability to respond to growth factors. While the affinity of tyrosine phosphorylated Spry2 for Cbl's TKB domain has not been compared directly to the affinity of Spry2 for the RING finger domain, our model predicts that Spry2 binds first to the Cbl's RING finger domain and is subsequently lured to the stronger interaction between Spry2 and Cbl's TKB domain. The differential affinity between these two modes of Spry2-Cbl interactions would explain why both the overexpression and the loss of expression of Spry2 can lead to overactive RTK signalling (Rubin et al., 2003; Fong et al., 2006). That is to say, overexpression of Spry2 leads to the inhibition of Cbl's E3 ubiquitin-ligase activity through its competition with E2 enzymes for binding to the RING finger domain, whereas, loss of Spry2 expression leads to the functional loss of Cbl's E3 activity by preventing Cbl from exchanging spent E2 enzymes for ubiquitin loaded E2 enzymes. Therefore, the deregulation or loss of Cbl-regulatory proteins such as Spry2, similar to the deletion or loss of regulatory amino acids within Cbl's Linker domain, could induce cellular transformation by promoting a dominant negative Cbl phenotype.

3.3.11 Wild-type Cbl and cancer

Cbl is commonly thought of as a negative regulator of cellular signalling because of its function in the downregulation of activated RTKs, as such, the study of wild-type Cbl as a positive correlator of cancer has not been well investigated. I have identified several possible scenarios in which the aberrant regulation of Cbl leads to cellular transformation: 1) transformation induced by mutations within Cbl's Linker domain, 2) transformation induced by the constitutive tyrosine phosphorylation of Cbl's Linker domain or 3) transformation induced by the loss of proteins that regulate Cbl's TKB domain-mediated protein-protein interactions with substrate proteins.

Cbl Linker domain mutations, similar to that of 70ZCbl, were recently found in both c-Cbl and Cbl-b in leukemic cells isolated from AML patients (Caligiuri et al., 2007). This discovery marks the first transforming Cbl mutations to be identified in humans. While it remains unknown whether these Cbl mutations were causative in the formation of AML in these patients, knockdown of the mutant Cbl proteins was sufficient to significantly decrease the rate of growth of AML cells expressing these proteins (Caligiuri et al., 2007). This discovery raises the possibility of using Cbl as a target for cancer therapy, made more broadly applicable to other cancers by the possibility that deregulation of endogenous wtCbl may also promote or aggravate cellular transformation.

Indeed, endogenous Cbl may contribute to carcinogenesis via constitutive tyrosine phosphorylation of the Linker domain induced by association with mutated or overexpressed transforming PTKs. This is supported by several studies examining Cbl expression levels, tyrosine phosphorylation status, and membrane association in human cancers: constitutively tyrosine phosphorylated c-Cbl has been found in many different human cancer tissue samples (Kamei et al., 2000) and Cbl expression levels have been correlated with poor prognosis in gastric carcinomas that also expressed high levels of EGFR (Ito et al., 2004). Also, supporting an earlier study that correlated v-Abl-mediated Cbl tyrosine phosphorylation with transformation in cell culture (Andoniou et al., 1994), Cbl was found to be constitutively tyrosine phosphorylated and membrane associated in BCR-Abl expressing cancer cells from CML patients (Brizzi et al., 1998).

As mentioned, Cbl may also become deregulated and transforming in the absence of proteins such as Spry2, which likely help to regulate Cbl's TKB domain-mediated protein-protein interactions. Spry2 loss has been correlated, in several different studies, with human cancers associated with deregulated RTKs (Lo et al., 2004; Fong et al., 2006; Fritzsche et al., 2006; Sutterluty et al., 2007). While no direct evidence exists to link Spry-loss with Cbl-mediated transformation, our model for Cbl-mediated transformation suggests that the loss of Cbl regulatory proteins effectively generates dominant negative Cbl, similar to the CblYYDD, by impairing the ability of Cbl to release from TKB domain binding partners and to replace spent E2 ubiquitin-conjugating enzymes.

This evidence, combined with my experimentally derived models for Cbl-mediated transformation and Cbl regulation, identify Cbl as a potential therapeutic target for the treatment of cancer by suggesting that constitutively tyrosine phosphorylated wtCbl and deregulated wtCbl, may promote and/or aggravate cellular transformation similar to transforming mutants of Cbl. As there are no known Cbl-directed therapeutic agents, further research in this area is merited.

3.3.12 Summary

We have generated a new model for Cbl-mediated transformation that fits all of the data available for the different naturally occurring and artificially generated transforming mutants of Cbl as well as the E3 ubiquitin-ligase null non-transforming mutants of Cbl. This model predicts that a transforming Cbl mutation would have to induce a loss-of function with respect to E3 ubiquitin-ligase activity and also induce a gain-of-function by increasing the affinity of the TKB domain for membrane associated Cbl-substrates. This type of mutation would give these Cbl mutants a competitive advantage over wtCbl in their ability to associate with TKB domain substrates, thus allowing them to effectively function as dominant negative proteins and induce cellular transformation.

I validate this hypothesis by demonstrating that mutations that can activate Cbl's TKB domain, when coupled with the non-transforming E3 ubiquitin-ligase null C381A mutation, can induce cellular transformation. I also demonstrate that TKB domain activating mutations are, on their own, sufficient to promote cellular transformation. This observation suggests that activating TKB domain mutations also abrogate E3 ubiquitin ligase activity *in vivo*. We propose that TKB domain activating mutations induce the functional loss of Cbl's E3 ubiquitin-ligase activity *in vivo* by preventing Cbl from being able to efficiently exchange spent E2 ubiquitin-conjugating enzymes for new ubiquitin-loaded E2 enzymes. This observation also implicates wtCbl, which is commonly hyperphosphorylated in human cancers and cancer cell lines, in cellular transformation by suggesting that it may contribute to the transformed phenotype, in its hyperphosphorylated state, by behaving in as dominant negative protein. I conclude that these data identify Cbl as a potential therapeutic target for the treatment of human cancers

associated with mutant or hyperphosphorylated wtCbl. As there are no known Cbl-directed therapeutics, these data also support the identification of therapeutic strategies that can interfere with Cbl's ability to transform cells.

Chapter Four: Oxidized Piceatannol Induces the Loss of Cbl and the Disruption of Oncogenic Cbl-associated Signalling Pathways.

4.1 Introduction

4.1.1 Current cancer therapeutics

Neoplastic transformation commonly arises from perturbations in cellular signalling pathways (Blume-Jensen and Hunter, 2001). Protein tyrosine kinases (PTKs) are tightly regulated signalling molecules that are essential components of many cell growth and survival signalling pathways. Genetic mutations that cause the disruption of the normal regulation of PTKs, including chromosomal translocations, gain of function mutations and gene amplifications, are commonly found in human cancers (Blume-Jensen and Hunter, 2001). The current trend in the development of cancer therapeutics is to target the individual PTKs that have been found to be misregulated in specific cancers. Successful applications of this strategy include Gleevec, which is used to inhibit Bcr-Abl in the treatment of chronic myelogenous leukemia (Mauro and Druker, 2001), Tarceva, which is used to specifically inhibit the epidermal growth factor receptor (EGFR) in the treatment of lung cancer (Johnson et al., 2005), and Herceptin, which is used to downregulate HER2 in the treatment of breast cancer (Meric-Bernstam and Hung, 2006). Many naturally occurring compounds have also been shown to inhibit a number of cancer associated kinases (Kingston and Newman, 2005; Beltz et al., 2006).

4.1.2 Piceatannol

Piceatannol is a small molecule that was initially isolated as the anti-leukemic agent from the domesticated oilseed *Euphorbia lagascae* (Ferrigni et al., 1984). In mammals and mammalian cell culture, piceatannol has also been shown to have beneficial effects as an anti-inflammatory agent (Ashikawa et al., 2002; Dang et al., 2004), an anti-histamine (Matsuda et al., 2004; Seow et al., 2004), and a general anti-cancer agent (Ferrigni et al., 1984; Wieder et al., 2001; Barton et al., 2004; Larrosa et al., 2004). Piceatannol is thought to accomplish these varied effects through its propensity to inhibit specific tyrosine and serine/threonine protein kinases. Piceatannol was initially shown to be, and is still commonly used as, a Syk-selective protein tyrosine kinase inhibitor (Geahlen and McLaughlin, 1989). It can also inhibit other tyrosine kinases,

including Src, Lck, and focal adhesion kinase (FAK), albeit with lower efficiency (Geahlen and McLaughlin, 1989; Thakkar et al., 1993; Law et al., 1999). More recently, piceatannol has been shown to inhibit several serine/threonine kinases (Wang et al., 1998; Youn et al., 2005). Piceatannol's cellular effects are not limited to kinase inhibition; it has been shown to induce apoptosis, which may be related to its inhibition of mitochondrial F_0F_1-ATPase activity (Zheng and Ramirez, 1999; Gledhill and Walker, 2005; Gledhill et al., 2007), and to induce DNA damage (Cavalieri et al., 2002; Hirakawa et al., 2002; Azmi et al., 2005). Piceatannol has also been shown to have antioxidant properties (Waffo Teguo et al., 1998; Ovesna et al., 2006).

4.1.3 Piceatannol: structure/function relationship

Piceatannol's structure has been implicated in each of its varied cellular effects. Piceatannol belongs to a class of molecules know as trans-stilbenes. The backbone of this class of molecule is composed of two phenyl rings connected by a two carbon methylene bridge (Figure 4.1). This structure is completely aromatic and as such, it is also planar. In addition to this basic structure, piceatannol has four hydroxyl groups, two per phenyl ring, located in a *meta* position on one ring and in the *ortho* position on the other. The placement of piceatannol's hydroxyl groups differentiates it from other polyphenolic trans-stilbenes, such as the highly similar compound resveratrol (Figure 4.1), and confers its specific properties (Thakkar et al., 1993; Larrosa et al., 2004; Ovesna et al., 2006). The combined effects of three of piceatannol's most characterized properties, namely, kinase inhibition, apoptosis induction and anti-oxidant activity, are responsible for its anti-cancer, anti-histamine and anti-inflammatory activity.

4.1.4 Piceatannol's properties and their cellular effects

4.1.4.1 Piceatannol is a kinase inhibitor

Piceatannol is most widely known as an inhibitor of protein tyrosine kinases. This property has been well characterized and is thought to be the result of piceatannol's structural similarity to tyrosine. Piceatannol has been shown to bind to the substrate binding pocket of the Syk protein tyrosine kinase, and it competitively inhibits this kinase by reducing its ability to associate with and phosphorylate its substrates (Geahlen and McLaughlin, 1989). While it has never been demonstrated experimentally, it is assumed

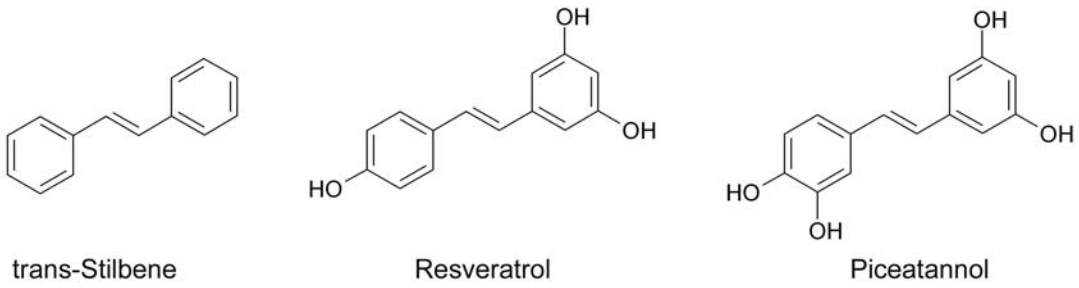


Figure 4.1 – Chemical structures of piceatannol and related trans-stilbenes.

that piceatannol's ability to inhibit other tyrosine kinases, including Lck, Src, and FAK, is also due to competitive inhibition of substrate binding (Geahlen and McLaughlin, 1989; Thakkar et al., 1993; Law et al., 1999). However, based on this mechanism of action, it is unclear how piceatannol is able to mediate the inhibition of serine/threonine kinases, as the substrate binding pockets of these proteins are not specific for aromatic amino acids.

Kinase inhibition has been suggested as the mechanistic basis for at least some of piceatannol's anti-cancer, anti-histamine, and anti-inflammatory effects. Piceatannol's anti-cancer effects have been linked to its ability to induce apoptosis, to its anti-oxidant activity, and to its ability to inhibit a number of kinases involved in cancer cell growth and survival signalling. Piceatannol-mediated inhibition of JAK1, a tyrosine kinase involved in the activation of STAT transcription factors, has been shown to sensitize non-Hodgkins lymphoma cell lines to cytotoxic chemotherapeutics agents by preventing the upregulation of anti-apoptotic proteins normally induced by STAT activation (Alas and Bonavida, 2003). In rat histiocytoma and human prostate cancer cell lines, piceatannol-mediated JAK/STAT inhibition has also been shown to induce apoptosis through downregulation of anti-apoptotic proteins (Barton et al., 2004; Kumari et al., 2005). Inhibition of Syk by piceatannol correlates with the induction of apoptosis in a number of human lymphoma cell lines, possibly by preventing the activation of mTOR and therefore inhibiting protein translation (Leseux et al., 2006; Rinaldi et al., 2006).

Piceatannol's anti-histamine activity has also been linked with its ability to inhibit Syk (Seow et al., 2004; Kepley, 2005), which is a prominent kinase in the histamine release pathway initiated by FcεRI activation (Kepley et al., 1998). Upon ligation of FcεRI, Syk becomes activated by the SFK Lyn. Activated Syk then mediates the tyrosine phosphorylation and recruitment of a number of adaptor proteins including SLP76, 3BP2, Cbl, and LAT. These phosphorylated adaptor proteins form a macromolecular signalling complex that recruits and activates PLC-γ, leading to PKC activation, Ca^{2+} mobilization, and subsequently, histamine release (Sada and Yamamura, 2003). Pharmacological inhibition or biological deficiency of Syk impairs the release of histamine in FcεRI stimulated mast cells and basophils (Seow et al., 2004; Kepley, 2005).

Piceatannol's anti-inflammatory activity has been associated with its ability to inhibit a number of kinases involved in the activation of the pro-inflammatory transcription factor NFκB (Ashikawa et al., 2002; Dang et al., 2004; Youn et al., 2005). Several studies have implied that piceatannol, and the highly related resveratrol affect NFκB activity by inhibiting the kinases IKKα and IKKβ (Holmes-McNary and Baldwin, 2000; Ashikawa et al., 2002), which are responsible for the phosphorylation-dependent degradation of the negative regulator of NF-κB, IκBα. However, no direct experimental evidence for the inhibition of these kinases was shown (Holmes-McNary and Baldwin, 2000; Ashikawa et al., 2002). Evidence opposing this model was provided in a study by Youn and colleagues, who demonstrated that resveratrol could not prevent NF-κB activation mediated by IKKα and IKKβ, but that it could block NF-κB activation through the direct inhibition of the kinases RIP1 and TBK1 (Youn et al., 2005). Studies published to date examining the two different proposed models of NFκB inhibition by piceatannol and/or resveratrol have yielded conflicting data. For example, Youn and colleagues showed that resveratrol had only limited ability to inhibit NF-κB activation as a result of Toll-like receptor signalling (Youn et al., 2005), compared with observations of Ashikawa and others who have reported a piceatannol- and resveratrol-induced inhibition of NF-κB activation resulting from a variety of different inflammatory stimuli including Toll-like receptors, TNFR, PMA, H_2O_2, and ceramide (Ashikawa et al., 2002). Considering that kinase inhibitors usually act in a very timely manner, and that Ashikawa and colleagues observed maximal inhibition after 8 hours of pre-treatment with piceatannol, it seems likely that this apparently conflicting data can be explained by the existence of at least two separate mechanism for the inhibition of NF-κB, with one pathway operating through inhibition of IKKα and IKKβ, and a separate pathway operating through inhibition of RIP1 and TBK1, depending on the specific cellular context and pro-inflammatory stimulus being used. This question remains the subject of debate and requires further study.

4.1.4.2 Piceatannol induces apoptosis

Piceatannol is known to be a potent inducer of apoptosis (Wieder et al., 2001; Barton et al., 2004; Larrosa et al., 2004). The mechanism(s) involved in this process are

not consistent and appear to be dependent upon the cell lines and experimental conditions used. This being the case, the induction of apoptosis has been attributed to several different properties associated with piceatannol including its inhibition of protein kinases, its inhibition of mitochondrial F_0F_1 ATPase activity, and its ability to cause DNA damage.

Piceatannol has been associated with the inhibition of several important kinases required for the survival of transformed cells, including Syk, Src, and JAK1. Syk inhibition in human lymphoma cell lines by piceatannol or curcumin has been shown to induce apoptosis (Rinaldi et al., 2006; Gururajan et al., 2007). Src inhibition has also been correlated with apoptosis in transformed cells in culture (Lee et al., 2004; Shor et al., 2007), and in mouse xenograft models (Yezhelyev et al., 2004). Inhibition of Jak1, using several different inhibitors, has also been shown to induce apoptosis (Sudbeck et al., 1999; Uddin et al., 2005). While these studies demonstrate a positive correlation between apoptosis and inhibition of piceatannol-sensitive kinases, the majority of the inhibitors used, including compounds such as PP2, dasatinib, and curcumin, have broad specificities (Lin, 2007; Rix et al., 2007) and it remains a possibility that the apoptosis observed in these studies was the result of off-target effects.

Piceatannol may also induce apoptosis through inhibition of mitochondrial F_0F_1 ATPase activity (Wieder et al., 2001; Gledhill et al., 2007). The mitochondrial F_0F_1 ATPase is a multi-subunit protein complex found in the inner mitochondrial membrane. This enzyme is responsible for converting the proton gradient generated by oxidative metabolism into energy in the form of ATP. Crystal structures of the F_1 subunit of the ATPase complex have demonstrated that piceatannol, and the related compounds resveratrol and quercetin, all associate with the same hydrophobic binding pocket. This interaction is mediated predominantly through van der Waals contacts and hydrogen bonds involving the compounds' hydroxyl groups (Gledhill et al., 2007). Binding of these compounds to the F_1 subunit prevents the ATPase from undergoing conformational changes required for its enzymatic activity (Gledhill and Walker, 2005; Gledhill et al., 2007). Inhibition of mitochondrial ATP synthesis has been shown to alter the mitochondrial membrane potential (Kalbacova et al., 2003), and both a reduction in

mitochondrial ATP production and alteration of the mitochondrial membrane potential have been linked to the induction of apoptosis (Skulachev, 2006). While this evidence supports a model where the inhibition of ATP synthesis by piceatannol induces apoptosis, it is still unclear whether the significant decrease in the mitochondrial membrane potential observed is due to piecaeatannol's inhibition of F_0F_1 ATPase activity or due to piceatannol's induction of apoptosis induced by separate signalling mechanisms (Wieder et al., 2001).

Piceatannol-induced apoptosis may also result from its ability to damage DNA through the formation of DNA adducts (Cavalieri et al., 2002) and DNA strand breaks (Hirakawa et al., 2002; Azmi et al., 2005). Piceatannol and related polyphenols generate DNA damaging hydroxyl radicals in the presence of transition metal ions, such as Cu^{2+} (Fukuhara and Miyata, 1998; Cavalieri et al., 2002; Hirakawa et al., 2002; Galati and O'Brien, 2004; Subramanian et al., 2004; Azmi et al., 2005). Planar molecules such as piceatannol, which can bind to DNA by intercalating between base pairs, can therefore generate potently DNA damaging hydroxyl radicals in close vicinity to DNA (Fukuhara and Miyata, 1998; Azmi et al., 2006). This property is highly dependent upon piceatannol's structure as both the number of hydroxyl groups and the rigidity of the molecule have been demonstrated to regulate the extent of DNA damage (Subramanian et al., 2004). The number of hydroxyl substituents found in polyphenolic compounds generally correlates with their antioxidant capacity (Murias et al., 2005), which has also been shown to be important in the induction of DNA damage. Piceatannol, which is a much more potent antioxidant than resveratrol due to the presence of an additional hydroxyl group (Murias et al., 2005), has been shown to induce comparatively more DNA damage than resveratrol (Azmi et al., 2005). While these studies convincingly demonstrate that piceatannol mediates DNA damage *in vitro*, *in vivo*, piceatannol's DNA damaging capabilities are much less clear, as contradictory reports indicate that piceatannol's antioxidant properties are both protective and damaging to DNA (Waffo Teguo et al., 1998; Johnson and Loo, 2000; Loo, 2003; Murias et al., 2005; Ovesna et al., 2006). One possible explanation for these differences is that as the concentration of piceatannol is increased its protective anti-oxidant effects are decreased: low

concentrations of piceatannol were shown to be protective towards radical-induced DNA damage (Loo, 2003; Ovesna et al., 2006), whereas higher concentrations were shown to promote DNA damage (Loo, 2003; Azmi et al., 2005; Azmi et al., 2006).

Its ability to induce apoptosis has led to consideration of piceatannol as an anticancer agent (Wieder et al., 2001; Barton et al., 2004; Larrosa et al., 2004). Currently there is no consensus regarding the mechanism(s) by which piceatannol is able to induce apoptosis. It is likely that, depending upon the treatment conditions used, more than one of the pro-apoptotic effects mediated by piceatannol, the inhibition of kinases involved in cell survival signalling (Wieder et al., 2001), the inhibition of mitochondrial ATP synthesis (Wieder et al., 2001), and the induction of DNA damage (Johnson and Loo, 2000; Cavalieri et al., 2002; Ahmad et al., 2005), are involved.

4.1.4.3 Piceatannol is an antioxidant

Piceatannol also possesses antioxidant properties. Due to its aromatic structure and the presence of multiple electron-rich hydroxyl groups, piceatannol is able to donate electrons to neutralize highly reactive free radicals (Ovesna et al., 2006). Unpaired electrons are delocalized throughout piceatannol's aromatic structure, generating stable semiquinones. Further electron donation is facilitated by the presence of ortho-hydroxyl groups that allow for the energetically favourable formation of even more stable o-benzoquinone species (Murias et al., 2005). Piceatannol's trans-stilbene backbone is also important for its potent antioxidant capacity, as this structure has been shown to be more thermodynamically favourable with respect to electron donation than the cis-conformation (Stivala et al., 2001). This is apparent when comparing the small amount of DNA damage produced by the cis-hydroxystilbenes used by Subramanian et al. (Subramanian et al., 2004), to the massive amount of DNA damage observed by Azmi and colleagues using the corresponding trans-hydroxystilbenes (Azmi et al., 2005).

Piceatannol's antioxidant capacity is thought to mediate anticancer properties through two conflicting mechanisms. As previously discussed, the antioxidant capacity of polyphenols positively correlates with their ability to induce DNA damage. Catechol-containing compounds, which have an increased antioxidant capacity resulting from their ability to form relatively stable o-benzoquinones (Murias et al., 2005), are more effective

than other polyphenolic compounds at inducing DNA damage (Hirakawa et al., 2002; Subramanian et al., 2004). Alternatively, it has been reported that the antioxidant capacity of piceatannol and related dietary polyphenols act to prevent cancer formation by reducing ROS induced DNA damage (Waffo Teguo et al., 1998; Ovesna et al., 2006).

Piceatannol's antioxidant capacity has also been hypothesized to mediate its anti-inflammatory properties by reducing the amount of inflammation-induced free radicals. These free radicals, in the form of NO and ROS, mediate vasodilation and microbial destruction, respectively, but also lead to tissue damage (Afonso et al., 2007). However, it is unlikely that a physiologically attainable concentration of piceatannol could neutralize a sufficient quantity of free radicals to see a noticeable reduction in inflammation, given the very high concentrations of free radicals produced in areas of localized inflammation.

4.1.4.4 Piceatannol induces Cbl loss

Despite the extensive characterization of its properties and cellular effects, piceatannol has never been associated with the loss of specific proteins. In this chapter, I demonstrate that piceatannol induces the loss of the c-Cbl proto-oncogene, as well as the Cbl-dependent loss of specific, Cbl-associated PTKs.

4.1.5 Cbl proteins

As discussed in Chapter Three, the c-Cbl proto-oncogene is an adaptor protein and a RING finger E3 ubiquitin-ligase; as such, it acts as both a positive and negative regulator of many RTKs and NRTKs. Upon activation of RTKs, c-Cbl is recruited from the cytosol to the activated receptor complex where it recruits signalling effector proteins and positively regulates signalling by acting as an adaptor to facilitate the formation of higher-order multi-component signalling complexes. Cbl then acts to ubiquitinate these receptors as well as many of their associated signalling proteins (Thien and Langdon, 2001). This causes them to be degraded by the proteasome and/or lysosome (Thien and Langdon, 2001). Cbl itself is also regulated by ubiquitin-mediated proteolysis (Thien and Langdon, 2001; Yokouchi et al., 2001; Howlett and Robbins, 2002; Magnifico et al., 2003). Thus, through its E3 ubiquitin-ligase activity, Cbl also functions as a potent negative regulator of protein tyrosine kinase signalling.

Cbl was originally identified based on its homology with the retrovirally encoded v-Cbl oncogene (Langdon et al., 1989). In addition, we and others have identified two naturally occurring transforming mutant isoforms of c-Cbl: p95Cbl and 70ZCbl (Andoniou et al., 1994; Bisson et al., 2002). These mutant c-Cbl proteins contain deletions of the RING finger, or of areas immediately proximal to the RING finger, which cripple their E3-ligase activity (Andoniou et al., 1994; Thien and Langdon, 2001; Bisson et al., 2002). While searching for inhibitors of Cbl-mediated transformation, I found that piceatannol dramatically reduced Cbl protein levels in many different cell lines.

4.1.6 Objectives

Cellular signalling pathways are misregulated and overactive in cancer cells. Many current cancer therapeutics aim to downregulate these oncogenic signalling pathways. While searching for compounds that could revert Cbl-mediated cellular transformation, I observed that piceatannol induced the loss of Cbl. In this chapter, I assess the mechanism of piceatannol-induced Cbl loss to determine the therapeutic potential of piceatannol as an anti-cancer agent. After demonstrating that this phenomenon was not due to the stimulation of known Cbl-regulatory proteolytic pathways, I proposed that this loss was mediated directly by piceatannol, through its oxidative conversion into an o-benzoquinone. I tested the validity of this hypothesis using a well-defined *in vitro* reaction system that recapitulated piceatannol-Cbl interactions under controlled conditions to enable dissection of the molecular mechanism of Cbl loss. I further characterized the protein selectivity of this process and established the applicability of these observations to cancer treatment. In summary, the work presented in this Chapter characterizes a previously unrecognized piceatannol-dependent cellular effect, and demonstrates a novel way in which this effect can be exploited as a cancer therapeutic to inhibit oncogenic signalling pathways.

4.2 Results

4.2.1 Piceatannol induces the loss of c-Cbl and mutant oncogenic Cbl proteins

c-Cbl has several tyrosine residues that are inducibly phosphorylated in response to extracellular stimuli under normal circumstances, or that are constitutively

phosphorylated in the transforming mutants of Cbl, 70ZCbl and p95Cbl (Andoniou et al., 1994; Bisson et al., 2002). While searching for compounds that could reverse Cbl-mediated transformation, I screened murine fibroblasts expressing p95Cbl with a broad range of tyrosine kinase inhibitors. Piceatannol, widely used as a Syk-selective tyrosine kinase inhibitor, consistently displayed the most dramatic effect on Cbl tyrosine phosphorylation levels (Bisson, 2004). I found that this was due to piceatannol treatment leading to a loss in Cbl protein levels, as detected by Western blotting (Figure 4.2) (Bisson, 2004). To determine if piceatannol was only able to induce the loss of p95Cbl, I also examined Cbl protein levels from piceatannol treated murine 3T3 fibroblasts overexpressing wtCbl, 70ZCbl, p95Cbl, v-Cbl, and Δ1-355Cbl, an amino-terminal truncation of Cbl. All of the Cbl proteins, wild type and mutant, were lost upon treatment with piceatannol, as demonstrated by Western blotting (Figure 4.2). These data indicated that piceatannol treatment of cells lead to the loss of Cbl protein, and that no specific region of Cbl made Cbl susceptible to piceatannol-induced degradation.

4.2.2 Piceatannol-induced Cbl loss is not due to a Western blotting artifact

To ensure that the loss of Cbl was not due to an artifact of the lysis or Western blotting procedures, duplicate plates of wtCbl overexpressing murine 3T3 fibroblasts were lysed in NP-40 lysis buffer or hot SDS sample buffer and both the soluble and pellet fractions of the NP-40 lysates were loaded on an SDS-PAGE gel along with the SDS lysates. The stacking gel was left intact and transferred to nitrocellulose along with the resolving gel to ensure Cbl was not lost due to an inability to enter into the resolving gel. Western blotting confirmed that Cbl was not being lost due to either lysis or Western blotting artifacts (Figure 4.3).

To ensure that the observed piceatannol-induced Cbl loss was not due to the modification or loss of the epitope recognized by the c-Cbl specific monoclonal antibody, I reprobed Western blots of HA-tagged 70ZCbl overexpressing murine 3T3 fibroblasts lysates with a polyclonal antibody directed towards 70ZCbl's carboxy-terminus as well as a monoclonal anti-HA antibody that recognized the HA-tag fused to 70ZCbl's amino-terminus. All three antibodies demonstrated that the epitope-tagged 70ZCbl was lost in a dose-responsive manner in cells treated with increasing amounts of piceatannol

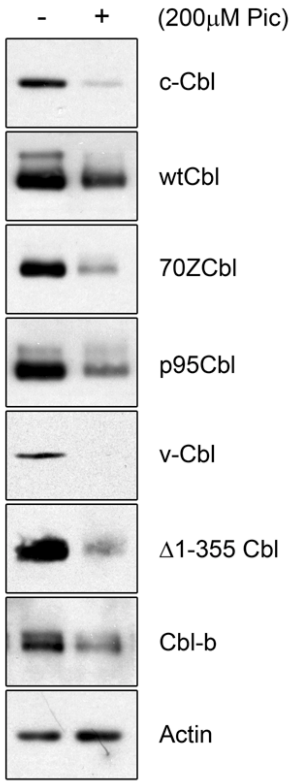


Figure 4.2 – Piceatannol treatment of cells induces Cbl loss.

Western blots of murine 3T3 fibroblasts stably expressing different Cbl proteins and treated for 3 hours with DMSO (-) or 200μM piceatannol (+). Lysates from vector control fibroblasts were used to detect endogenous c-Cbl, Cbl-b, and actin as a representative loading control. Lysates from cells overexpressing wild-type c-Cbl (wtCbl), 70ZCbl, p95Cbl, Δ1-355Cbl, and v-Cbl were probed for Cbl-levels to determine which regions of c-Cbl were sensitive to piceatannol treatment. (Representative of n=3)

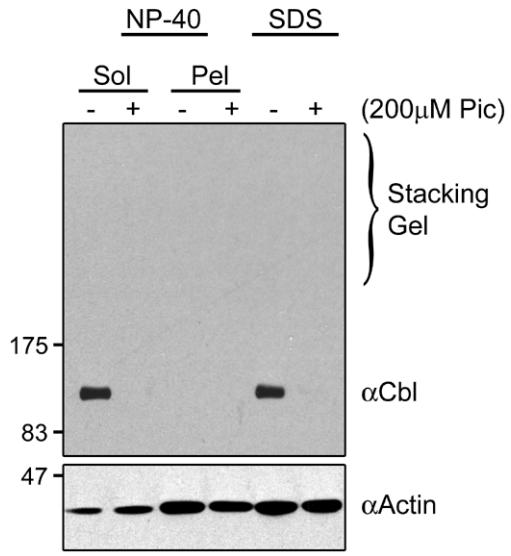


Figure 4.3 – Piceatannol-induced Cbl-loss is not a Western blotting artifact.
Western blots of wtCbl expressing murine 3T3 fibroblasts treated with 200μM piceatannol or DMSO and lysed in 1% NP-40 buffer or SDS sample buffer. Both the soluble and pellet fraction of the NP-40 lysates are included. The stacking gel was transferred to ensure that Cbl was not being lost due to its inability to enter into the resolving gel. (Representative of n=2)

(Figure 4.4).

4.2.3 Piceatannol-induced Cbl loss is not due to changes in transcription

The piceatannol-induced loss of Cbl protein levels visualized by Western blotting, while visible at time points earlier than 30 minutes, was most dramatic after 3 or more hours (Bisson, 2004). This time-frame for piceatannol-induced loss suggested the possibility that this effect could be at least partially mediated at a transcriptional level. To examine this possibility I isolated RNA from 70ZCbl expressing fibroblasts at 1, 3, 8, and 24 hours following piceatannol treatment. RT-PCR for *Cbl*, normalized to the loading control GAPDH, showed no decrease in the level of *Cbl* transcript in piceatannol treated cells (Figure 4.5). These data indicated that the observed reduction of Cbl was not due to piceatannol-induced changes in *Cbl* transcription or *Cbl*-transcript stability. Together these data suggested that piceatannol is mediating its effect on Cbl at the protein level.

4.2.4 Piceatannol-induced apoptosis is not responsible for decreasing Cbl protein levels

To determine the mechanism by which piceatannol was inducing Cbl protein loss, I examined piceatannol's ability to induce Cbl loss in the presence of inhibitors of the recognized cellular mechanisms of Cbl protein regulation: apoptosis-induced caspase-dependent degradation, ubiquitin-dependent proteasomal degradation and ubiquitin-dependent lysosomal degradation.

As piceatannol treatment has been shown to induce apoptosis in several different cell lines (Wieder et al., 2001; Barton et al., 2004; Larrosa et al., 2004), I confirmed piceatannol's ability to induce apoptosis in 70Z/3 cells. 70Z/3 cells, the mouse pre-B cell lymphoma cell line from which 70ZCbl was initially cloned (Andoniou et al., 1994), were used instead of 70ZCbl overexpressing mouse fibroblasts as these cells were more amenable to the analysis of apoptotic markers. I examined two different apoptotic markers to evaluate whether piceatannol induced apoptosis in 70Z/3 cells. First, I used flow cytometric analysis of 70Z/3 cells to look for phosphatidylserine in the outer leaflet of the plasma membrane using FITC-labelled annexinV (Figure 4.6, graph). This data correlated well with Western blots examining the cleavage of a second apoptotic marker (Figure 4.6, bottom panels), PARP, which is directly targeted and cleaved by activated

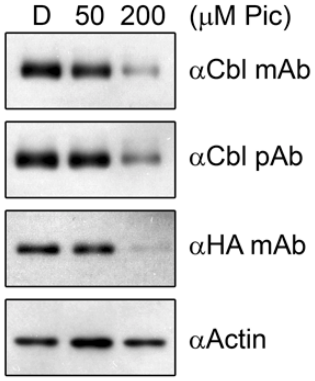


Figure 4.4 – Piceatannol-induced Cbl loss is not due to epitope loss.

Western Blots from lysates of 70ZCbl-HA overexpressing murine 3T3 fibroblast cells treated for 3 hours with DMSO (D), 50μM, or 200μM piceatannol. Replicate blots were probed using a c-Cbl specific monoclonal antibody (Cbl mAb), a c-Cbl specific polyclonal antibody (Cbl pAb), an HA specific monoclonal antibody (HA mAb), or an actin specific monoclonal antibody (Actin) as a loading control. (Representative of n=4)

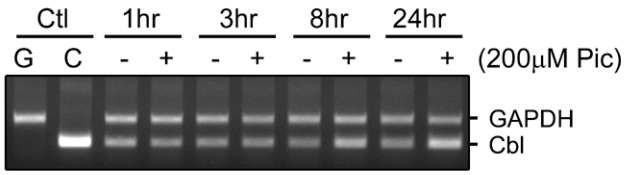


Figure 4.5 – Piceatannol-induced Cbl loss is not due to changes in Cbl transcription. RT-PCR, using RNA isolated from wtCbl expressing murine 3T3 fibroblasts, demonstrating no decrease in the level of Cbl mRNA as a result of 200μM piceatannol (+) treatment compared to DMSO treatment (-), after 1,3,8, and 24 hours. (Representative of n=3)

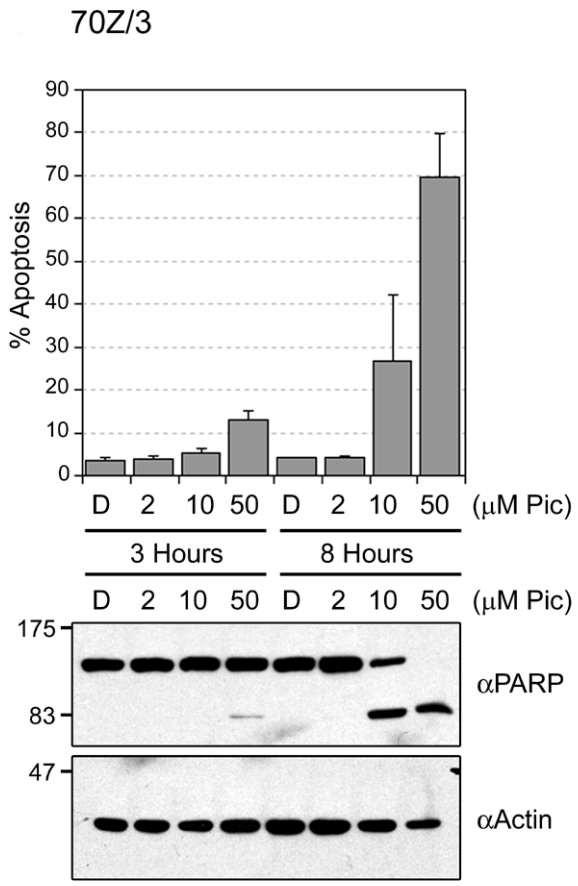


Figure 4.6 – Piceatannol induces apoptosis in 70Z/3 cells.

Quantification of apoptotic 70Z/3 cells after treatment with DMSO (D), 2μM, 10μM, or 50μM piceatannol for 3 and 8 hours. The top panel indicates the percentage of cells undergoing apoptosis as determined by annexin V-FITC and propidium iodide staining. Cells were considered apoptotic if they stained positive for annexin V alone or stained for both annexin V and propidium iodide. The bottom panel indicates apoptotic cells by Western blotting for PARP (~115kDa) and its caspase-cleaved apoptotic fragment (~85kDa). (Representative of n=3, except the flow cytometry assay n=4)

caspases during apoptosis (Nicholson et al., 1995). Both markers demonstrated that piceatannol induced apoptosis in 70Z/3 cells in a dose- and time-dependent manner.

Since piceatannol-induced apoptosis was concurrent with the reduction of Cbl levels, and apoptosis-induced caspase activation has been shown to induce the degradation of Cbl (Widmann et al., 1998), I examined whether the loss of Cbl was due to the apoptotic event and specifically whether it was due to the activation of caspases. In order to assess this I first treated 70Z/3 cells with a number of agents that promote apoptosis, including: hydrogen peroxide, UV radiation, and gamma-radiation. All of the agents induced the caspase-mediated cleavage of PARP in the 70Z/3 cells; however, only piceatannol was able to induce the loss of Cbl (Figure 4.7). This suggested that apoptosis was not the cause of piceatannol-induced Cbl loss.

To specifically examine the potential role of caspases in piceatannol-mediated Cbl loss, I treated 70Z/3 cells with the broad spectrum caspase inhibitor Boc-D-FMK prior to the addition of piceatannol. While the use of this caspase inhibitor produced a dose responsive inhibition of piceatannol-induced PARP cleavage, it was unable to prevent the reduction of Cbl protein levels (Figure 4.8). Together, these data indicated that piceatannol-induced Cbl loss was independent of caspase activation and confirmed that this loss does not require piceatannol-induced apoptosis.

4.2.5 c-Cbl is not ubiquitinated in response to piceatannol treatment

A distinct smearing of Cbl proteins was often observed on overexposed Western blots following exposure to piceatannol (Figure 4.9, right panel). This suggested that piceatannol was inducing a post-translational modification of Cbl. In its role as an E3-ubiquitin ligase, c-Cbl is involved in the targeting of receptors and cytoplasmic signalling molecules for degradation *via* the proteasomal and lysosomal pathways by mediating their multi- and poly-ubiquitination, respectively (Thien and Langdon, 2001). There is evidence that c-Cbl itself is destroyed in a ubiquitination-dependent manner (Yokouchi et al., 2001; Howlett and Robbins, 2002; Magnifico et al., 2003) and it is conceivable that piceatannol promotes this event. To examine whether piceatannol was inducing the ubiquitination of Cbl, I pre-treated wtCbl-overexpressing murine fibroblasts, transfected with HA-tagged ubiquitin, with the proteasome inhibitor MG132 prior to piceatannol

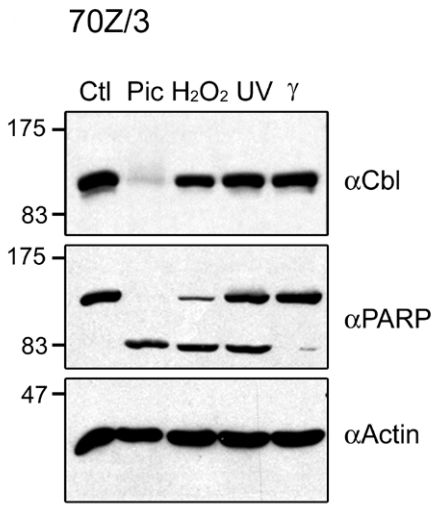


Figure 4.7 – Apoptosis induced by various stimuli does not mimic piceatannol-induced Cbl-loss.

Western blots from lysates of 70Z/3 cells treated for 6 hours with nothing (Ctl), 50μM piceatannol (Pic), 50μM H_2O_2, 100J UV (UV), or 6 Gray gamma-radiation (γ) to induce apoptosis. PARP cleavage indicates the induction of apoptosis in the absence of any noticeable Cbl degradation for all treatments except piceatannol. (Representative of n=3)

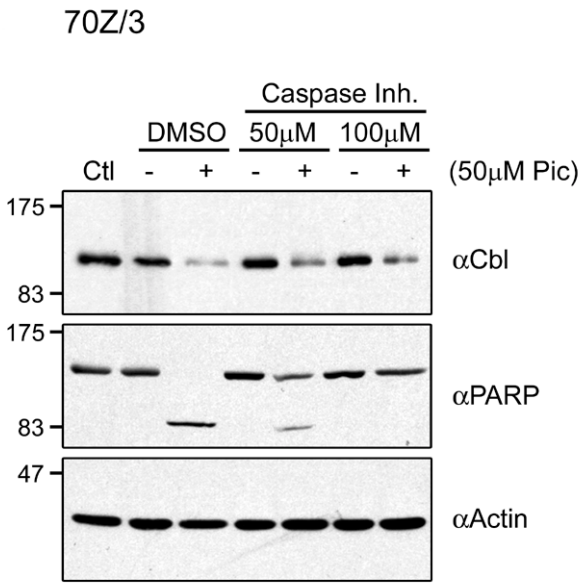


Figure 4.8 – Caspase activity is not required for piceatannol-induced Cbl-loss.
Western blots from 70Z/3 cells treated for 6 hours with DMSO (-) or 50μM piceatannol (+) after pretreatment for 2 hours with the indicated concentrations of the caspase inhibitor Boc-D-FMK (C). The dose dependent decrease in PARP cleavage indicates the effectiveness of the caspase inhibitor. (Representative of n=3)

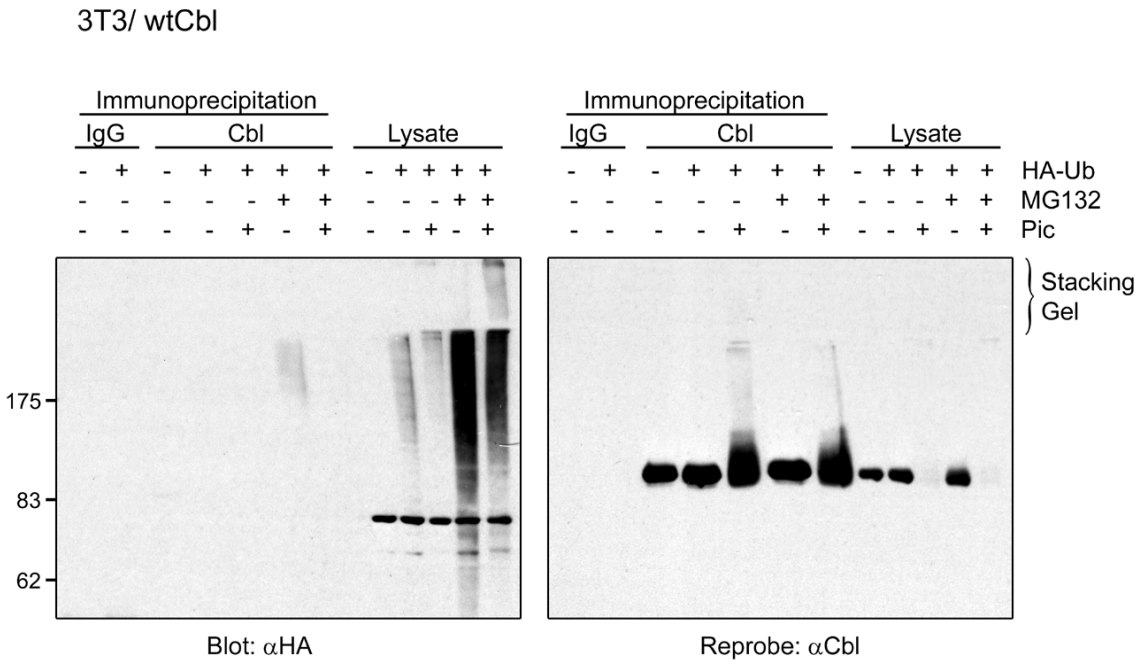


Figure 4.9 – Piceatannol does not induce Cbl ubiquitination.

Western blots of lysates and Cbl-immunoprecipitations from wtCbl expressing murine 3T3 fibroblasts transiently transfected with HA-tagged ubiquitin. Cells were treated with 200μM piceatannol (Pic) for 3 hours after a 4 hour pretreatment with the proteasomal inhibitor MG132 (25μM). Cbl was immunoprecipitated from the cell lysates with control IgG or the 7G10 monoclonal antibody. Western blots were probed for HA-ubiquitin using the 12CA5 anti-HA antibody (left panel) and reprobed for Cbl using a rabbit polyclonal antibody (right panel). The stacking gel was left on the Western blot to ensure that Cbl and ubiquitinated Cbl were not being lost due to an inability to enter into the resolving gel. (Representative of n=2)

treatment. By Western blotting with an anti-HA antibody, I could only detect HA-ubiquitin on Cbl immunoprecipitated from cells treated with MG132 alone (Figure 4.9 left panel). Reprobing these Western blots for Cbl (Figure 4.9, right panel) also demonstrated that MG132 did not induce the same pattern of Cbl-smearing as seen after piceatannol treatment. These results indicated that piceatannol treatment of cells does not lead to the ubiquitination of Cbl.

4.2.6 Proteasomal degradation is not responsible for piceatannol-induced Cbl loss

To confirm that piceatannol-induced ubiquitination of Cbl was not responsible for Cbl degradation, I further tested whether inhibition of the proteasome with pharmacological inhibitors could prevent the piceatannol-induced loss of 70ZCbl. Pre-incubation of 70ZCbl-overexpressing murine fibroblasts with the proteasome inhibitors ALLN, MG132, and epoxomycin, was not sufficient to prevent the piceatannol-induced loss of 70ZCbl (Figure 4.10). The pharmacological agents were used at effective doses, as proteasome inhibition was detected by the presence of ubiquitin laddering (Figure 4.10, lower panel). These data indicated that piceatannol did not induce Cbl loss *via* the proteasome and further confirmed that Cbl ubiquitination was not involved in this process.

4.2.7 Lysosomal degradation is not responsible for piceatannol-induced Cbl loss

Similar to what was observed after pretreating cells with proteasomal inhibitors, the pretreatment of cells with lysosomal inhibitors did not prevent the piceatannol-induced loss of Cbl. Bafilomycin A1 and ammonium chloride, inhibitors of lysosomal acidification that prevent lysosomal protease activation, as well as EST, an inhibitor of lysosomal cysteine proteases, were not capable of preventing the loss of 70ZCbl following piceatannol treatment (Figure 4.11). The lysosomal inhibitors were used at effective concentrations, as demonstrated by the 60% reduction in fluorescence of the lysosomal protease substrate $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 (Figure 4.11 bottom). These results demonstrate that lysosome-mediated protein degradation was not responsible for the piceatannol-mediated loss of c-Cbl proteins. Taken together with the data presented in sections 4.2.2 to 4.2.6, these results suggested that a novel and previously uncharacterized Cbl regulatory mechanism was induced upon treatment with piceatannol.

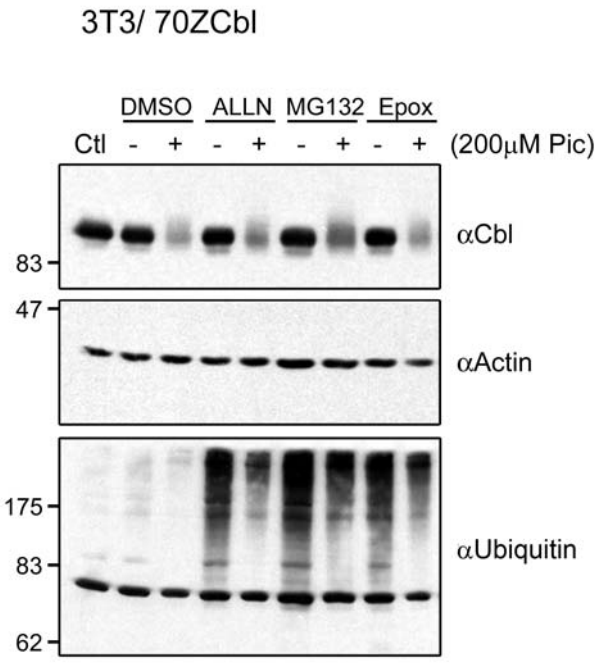


Figure 4.10 – Proteasomal degradation is not involved in piceatannol-induced Cbl-loss.

Western blots from lysates of 70ZCbl expressing murine 3T3 fibroblasts treated with 200μM piceatannol (Pic) for 3 hours after a 4 hour pretreatment with DMSO or the proteasomal inhibitors ALLN (10μM), MG132 (25μM), epoxomycin (200nM). The upper panel was probed for Cbl, the middle panel was probed for actin as a loading control, and the lower panel was probed for ubiquitin to demonstrate the effectiveness of the proteasomal inhibitors. (Representative of n=3)

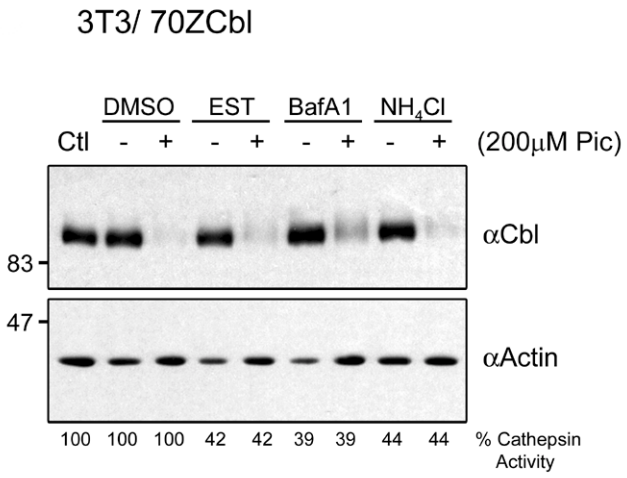


Figure 4.11 – Lysosomal degradation is not involved in piceatannol-induced Cbl-loss.

Western blots probed for Cbl (top panel) or actin (bottom panel) from lysates of 70ZCbl expressing murine 3T3 fibroblasts treated with 200μM piceatannol (Pic) for 3 hours after a 4 hour pretreatment with DMSO or the lysosomal inhibitors EST (25μM), bafilomycin A1 (200nM), and ammonium chloride (20mM). The numbers under the blots indicate the relative percentage of combined cathepsin B and cathepsin L protease activity in each experimental condition, prior to the addition of piceatannol, as measured by a fluorescent cathepsin substrate $(CBZ\text{-}Phe\text{-}Arg)_2$–R110 and quantified by flow cytometry. (Representative of n=3, except the cathepsin activity assay n=5)

4.2.8 Piceatannol treatment induces Cbl loss independently of cellular machinery

Many other proteases exist in cells other than those involved in apoptosis, the proteasome, and the lysosome. To test whether other proteases were involved in piceatannol-induced Cbl loss, I treated 70ZCbl overexpressing murine 3T3 fibroblasts with piceatannol and incubated them for 3 hours at 37°C or at 4°C. At 4°C, most enzymes, including cellular proteases, have minimal activity. Western blots of cellular lysates demonstrated that piceatannol induced Cbl loss at 4°C, in the absence of most cellular enzymatic activity (Figure 4.12), which implied that piceatannol was directly mediating loss of Cbl.

4.2.9 Kinase inhibition is insufficient to induce Cbl loss

To further confirm that piceatannol was not using cellular machinery to induce Cbl loss, I next examined the properties attributed to piceatannol to see which, if any, were involved in reducing Cbl protein levels. These properties include piceatannol's ability to inhibit kinases, to induce apoptosis, and to act as an anti-oxidant. As I had already examined and excluded piceatannol's ability to induce apoptosis as a cause for Cbl loss, I next examined whether kinase inhibition was involved. To determine if Cbl loss was due to piceatannol's ability to inhibit kinases, I treated 70ZCbl-overexpressing murine fibroblasts with a wide variety of tyrosine and serine/threonine kinase inhibitors. After a 3 hour treatment, only piceatannol was observed to induce a dramatic decrease in amount of Cbl detected without affecting actin levels (Figure 4.13). This result indicated that kinase inhibition was not sufficient to reproduce the dramatic loss of Cbl observed upon piceatannol treatment.

To further confirm this result, I then determined if the tyrosine phosphorylation status of c-Cbl played a role in piceatannol-induced Cbl loss. The K562 human erythroleukemia cell line was used to test this possibility, as these cells exhibit Cbl loss upon treatment with piceatannol and they contain high levels of constitutively tyrosine-phosphorylated c-Cbl, due to the presence of the Bcr-Abl oncogene, (Andoniou et al., 1994; Ribon et al., 1996). After an 8 hour pre-treatment with the Abl tyrosine kinase inhibitor STI571, c-Cbl tyrosine phosphorylation levels in the K562 cells were reduced to undetectable levels (Figure 4.14, upper panel). A further 12 hour treatment of these cells

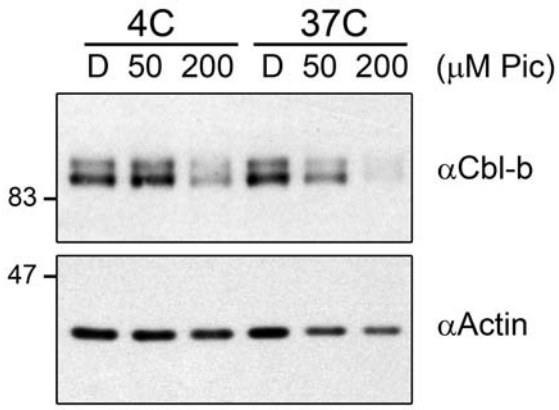


Figure 4.12 – Piceatannol treatment induces Cbl-loss independently from cellular proteases.

Western blots probed for Cbl-b (top panel) or actin (bottom panel) from lysates of 70ZCbl expressing murine 3T3 fibroblasts treated with DMSO (D), 50μM, or 200μM piceatannol for 3 hours at either 4°C or 37°C. (Representative of n=2)

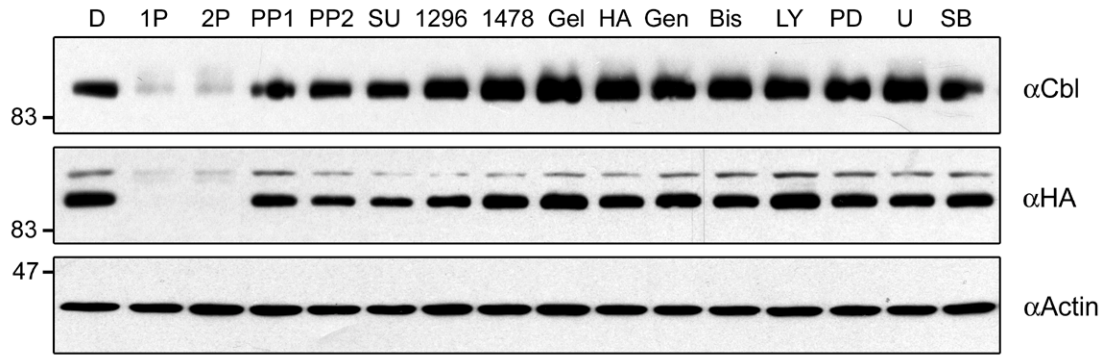


Figure 4.13 – Piceatannol, unlike other kinase inhibitors, induces Cbl-loss.
HA-70ZCbl expressing murine 3T3 fibroblasts were treated for 3 hours with DMSO (D) or with several tyrosine and serine/threonine kinase inhibitors including 100μM (1P) and 200μM (2P) piceatannol, 10μM PP1, 10μM PP2, 10μM SU6656 (SU), 10μM AG1296 (1296), 10μM AG1478 (1478), 2.5μM geldanamycin (Gel), 1μM herbimycin A (HA), 50μM genistein (Gen), 2μM bisindolylmaleimide I (Bis), 10μM LY294002 (LY), 25μM PD98059 (PD), 10μM U0126 (U), and 10μM SB203580 (SB). Western blots of lysates were probed for the N-terminal HA-tag (center panel) as well as for the C-terminal 7G10 epitiope (top panel). (Representative of n=2)

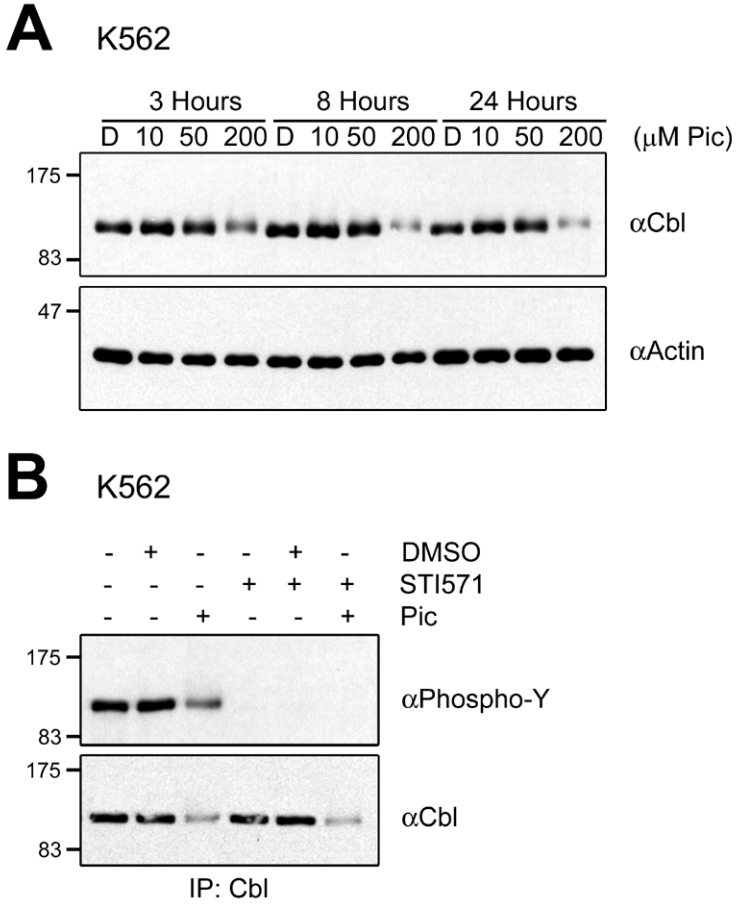


Figure 4.14 – The tyrosine phosphorylation status of Cbl does not affect its sensitivity to piceatannol.

A Western blots of K562 cells treated with DMSO, 10μM, 50μM, or 200μM piceatannol for the increasing amounts of time demonstrating that piceatannol treatment leads to Cbl-loss in these cells. The upper panel is probed for Cbl and the lower panel is probed for actin. **B** Western blots of Cbl immunoprecipitations from K562 cells pre-treated for 8 hours with 1 μM STI571 and then treated with 200μM piceatannol (Pic) or DMSO for a further 12 hours. The upper panel is probed with an anti-phosphotyrosine antibody, while the lower panel is probed for Cbl. (Representative of n=3)

with piceatannol resulted in a reduction in the amount of c-Cbl protein that was comparable to that observed in cells treated with piceatannol alone (Figure 4.14, lower panel). These data indicated that the tyrosine phosphorylation status of c-Cbl did not play a significant role in its sensitivity to piceatannol and further confirmed that the piceatannol-mediated inhibition of kinase activity was not involved in Cbl loss.

4.2.10 Antioxidant properties are not sufficient to induce Cbl loss

Piceatannol's antioxidant capacity was the final property of piceatannol that I examined to determine if it was involved in piceatannol-induced Cbl loss. This was accomplished by comparing the piceatannol treatment of 70ZCbl-overexpressing murine fibroblasts with several different antioxidants, including N-acetyl cysteine, as well as the natural products resveratrol, curcumin, and epigallocatechin gallate. Western blot analysis indicated that only piceatannol was able to induce Cbl loss (Figure 4.15); demonstrating that antioxidant properties were not sufficient to induce Cbl loss.

4.2.11 Resveratrol, a related trans-stilbene, was unable to mediate the loss of Cbl proteins

Resveratrol is a piceatannol-related polyphenolic compound with anticancer activity (Ulrich et al., 2005). Both piceatannol and resveratrol share a trans-stilbene backbone but differ in the number of hydroxyl substitutions (Figure 4.1). It has been suggested that resveratrol's anti-tumour activity is based on its conversion to piceatannol by the cytochrome P450 enzyme CYP1B1 (Potter et al., 2002), an enzyme that is overexpressed in a variety of human tumours (Potter et al., 2002). While I had already compared piceatannol treatment to resveratrol treatment and demonstrated that resveratrol had no effect on Cbl protein levels, it was possible that the CYP1B1 enzyme was unable to convert resveratrol to piceatannol in sufficient quantities at the 3 hour time point tested. Therefore, I investigated whether treatment of several different cell lines over an extended time course with resveratrol, or trans-stilbene, could elicit an effect similar to piceatannol on Cbl protein levels. As shown in Figure 4.16A, neither resveratrol treatment nor trans-stilbene treatment of mutant Cbl overexpressing mouse fibroblast cells was able to induce a reduction of Cbl protein levels, while piceatannol treatment potently promoted the loss of 70ZCbl. Similar results were obtained from the

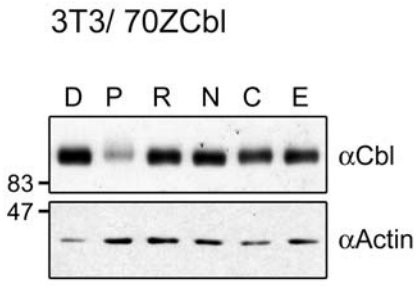


Figure 4.15 – Antioxidant properties are not sufficient to induce Cbl-loss.

70ZCbl expressing murine 3T3 fibroblasts were treated for 3 hours with DMSO (D), 200uM piceatannol (P), 200uM resveratrol (R), 5mM N-acetyl cysteine (N), 50uM curcumin, or 200uM epigallocatechin gallate (E). Western blots of cell lysates were probed for Cbl (top panel) or actin (bottom panel). (Representative of n=3)

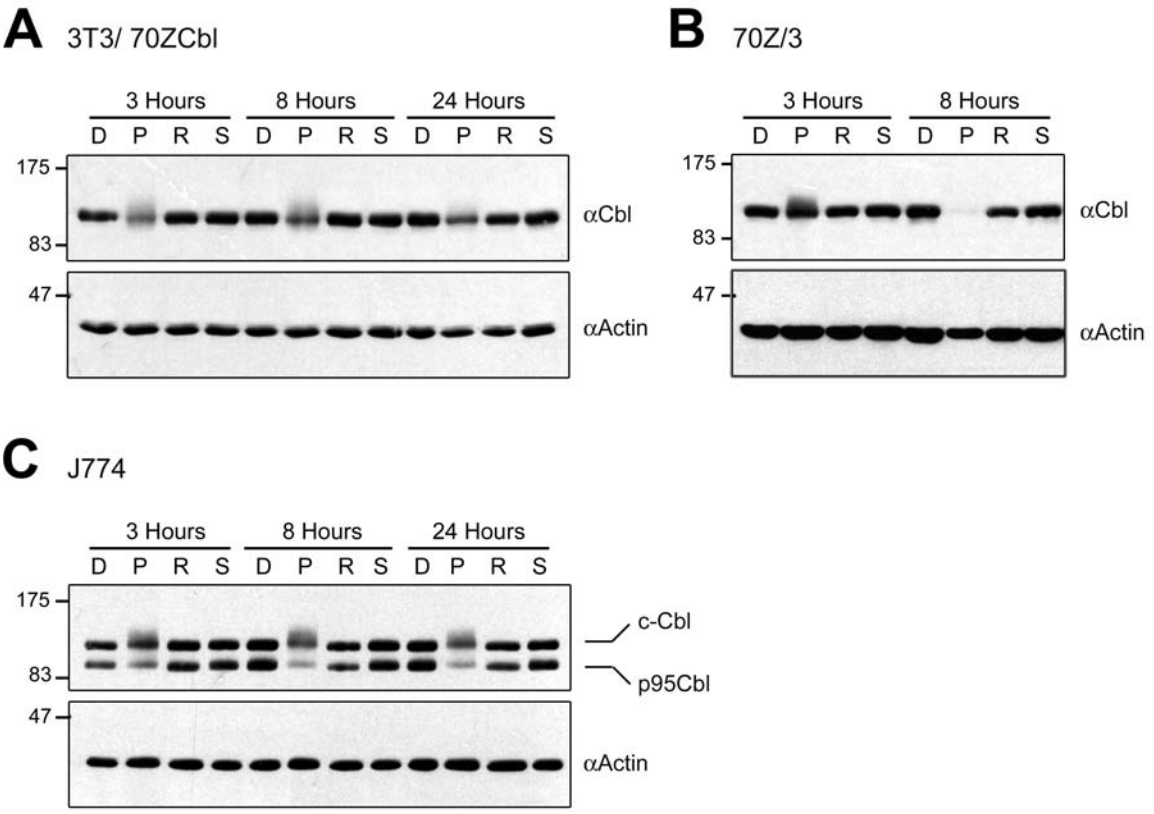


Figure 4.16 – Piceatannol, but not the related trans-stilbene resveratrol, leads to the loss of Cbl.

Western blots probed for Cbl (top panels) and actin (bottom panels). **A** 70ZCbl expressing murine 3T3 fibroblasts, **B** 70Z/3, and **C** J-774 cells treated with DMSO (D), 200μM piceatannol (P) or 50μM piceatannol for the 70Z/3 cells, 200μM resveratrol (R), or 200μM trans-stilbene (S) for 3, 8, or 24 hours. (Representative of n=3)

treatment of the 70Z/3 murine pre-B cell lymphoma cell line and the J-774A.1 murine reticulum cell sarcoma cell line (Figure 4.16B and 4.16C), which are the cell lines from which 70ZCbl and p95Cbl, respectively, were initially cloned (Andoniou et al., 1994; Bisson et al., 2002). These results indicated that, despite its structural similarity with piceatannol, resveratrol was incapable of producing the observed reduction of Cbl protein levels induced by piceatannol.

4.2.12 Aromatic catechol-containing compounds with limited degrees of freedom induced Cbl loss

My results indicated that piceatannol was inducing the loss of Cbl in treated cells independently of previously characterized Cbl-protein regulatory mechanisms and independently of cellular enzymes, and that Cbl loss was due to a heretofore unrecognized property of piceatannol. They also indicated that the structurally similar compound, resveratrol, was unable to mediate this effect. To gain a better understanding of the mechanism by which piceatannol was inducing Cbl loss, I screened a number of piceatannol-like compounds in an attempt to determine the chemical structure(s) required to induce Cbl loss. The piceatannol-like molecules used had many chemical and structural similarities to piceatannol including aromaticity, size, the presence of hydroxyl groups, and hydrophobicity (Figure 4.17). 70Z/3 cells where used to screen the compounds, as these cells had demonstrated the highest degree of sensitivity to piceatannol-induced Cbl loss. The cells were incubated with 100 μM of each compound for 1 hour, to avoid artifacts due to the induction of apoptosis, and cell lysates were examined for Cbl protein levels by Western blotting (Figure 4.18). Compounds 7, 29, 31, and 33 were all able to induce Cbl loss. However, only compounds 7, 29, and 31 were able to induce the loss of Cbl without affecting actin protein levels, as was the case with piceatannol (Figure 4.18). The common feature of these chemical compounds was the presence of a catechol ring structure. In piceatannol and in compound 29, the catechol rings are unprotected, whereas in compounds 7 and 31, the catechol rings are protected by acetate esters. As cells contain numerous esterase enzymes, once these compounds enter into cells, they are de-esterified, exposing the catechol ring. The other common feature of these compounds is that they are all small aromatic molecules with limited

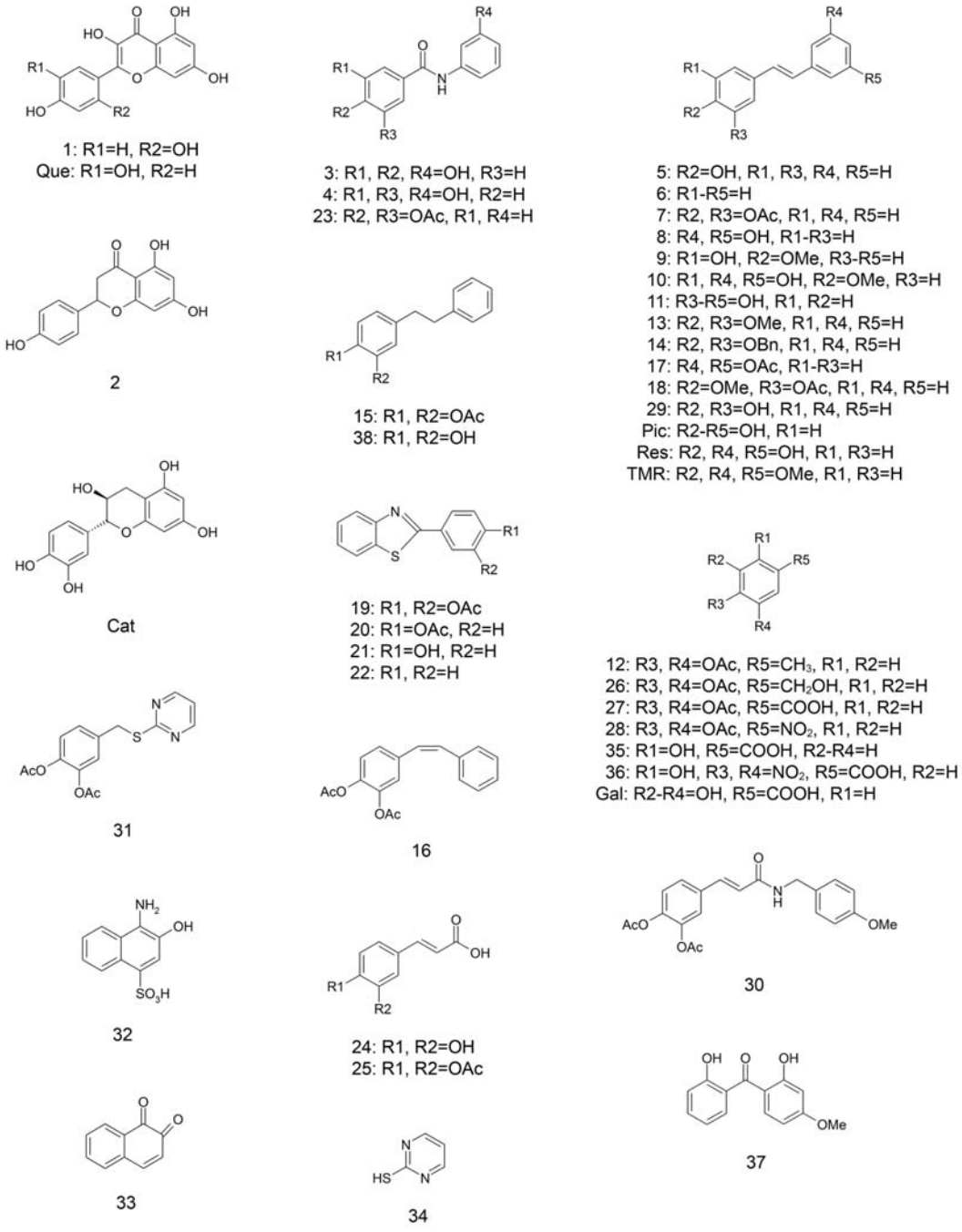


Figure 4.17 – Schematic of piceatannol-like molecules screened for the ability to induce Cbl loss.

Molecules are labeled by compound number or by abbreviation: piceatannol (Pic), resveratrol (Res), trismethoxy resveratrol (TMR), gallic acid (Gal), (+)-catechin (Cat) and quercetin (Que).

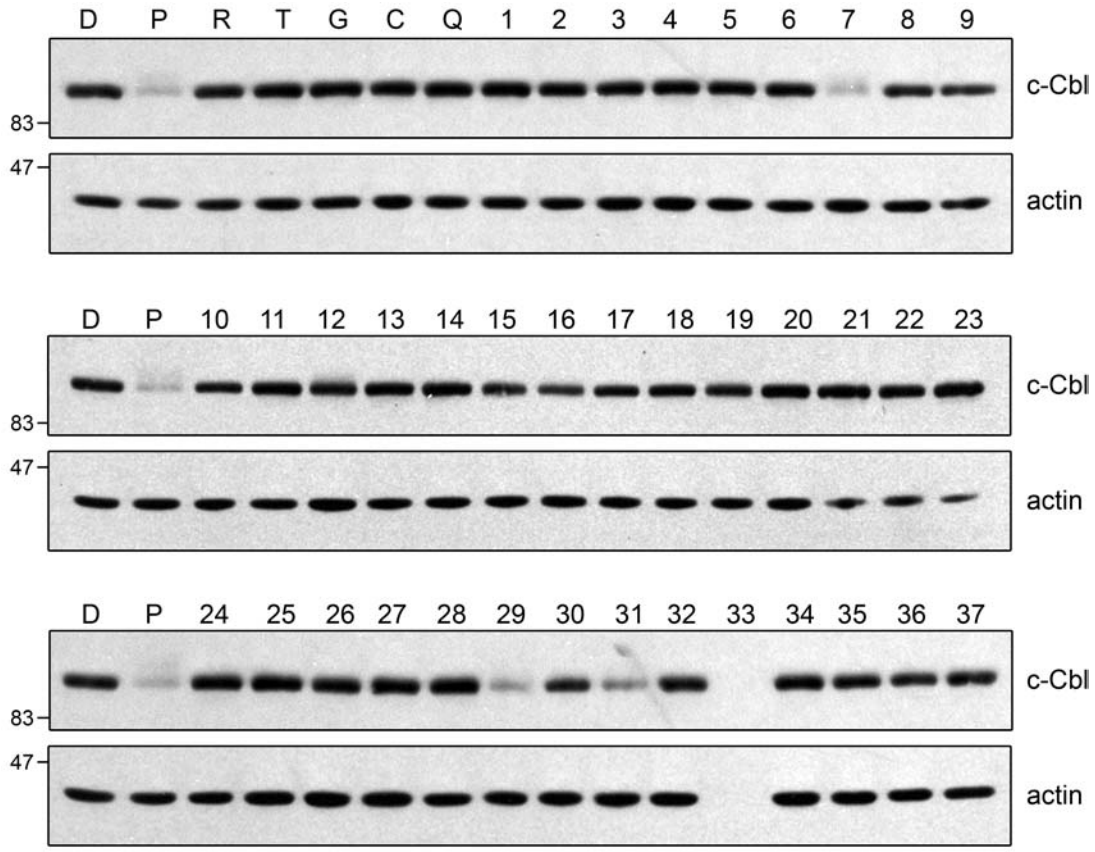


Figure 4.18 – Cell-based screen of piceatannol-like molecules for the ability to induce Cbl loss.

Western blots of lysates from 70Z/3 cells treated for 1 hour with DMSO (D) or 100μM of the indicated compounds: piceatannol (P), resveratrol (R), trismethoxy resveratrol (T), gallic acid (G), (+)-catechin (C), quercetin (Q), or compound 1-37 (#). Western blots were probed for c-Cbl (top panels) and actin (bottom panels).

degrees of freedom. Compounds 7 and 29, are planar trans-stilbenes like piceatannol, while compound 31 has only limited rotational freedom around its sulphur group. While other compounds in the screen possess these features individually, only piceatannol and these three compounds possess them in combination, which suggested that these particular structural properties are indispensable for the induction of Cbl loss.

4.2.13 Piceatannol directly reacts with purified Cbl, leading to the loss of Cbl protein

Due to the requirement for a catechol ring, and the observation that treatment of cells with piceatannol and compound 7 led to the media and cell pellets acquiring a brown colour after treatment (Boekelheide et al., 1979; Boekelheide et al., 1980), I hypothesized that oxidation of the catechol ring was involved in piceatannol-induced Cbl loss. This hypothesis also explains the observed smearing and loss of Cbl as the result of oxidized piceatannol causing protein cross-linking (Waidyanatha et al., 1998; Boatman et al., 2000) and chemical scission of the peptide backbone (Dean et al., 1997) (Figure 4.19). To test this hypothesis, I designed an *in vitro* assay system to test the minimal requirements for piceatannol-induced Cbl loss. Immunoprecipitated Cbl was resuspended in PBS and aliquoted into several different reaction mixtures containing combinations of trans-stilbene, H_2O_2, and HRP. The H_2O_2 and HRP were included to serve as a source of ROS to promote the oxidation of piceatannol and the other trans-stilbenes. After 15 minutes, the ROS in the reaction mixtures were neutralized by the addition of Laemmli's buffer containing β-mercaptoethanol, and the samples were analyzed by Western blotting. Only the combination of piceatannol, H_20_2, and HRP was able to induce specific Cbl loss, without affecting the levels of IgG heavy chain from the 7G10 antibody used to immunoprecipitate Cbl (Figure 4.20). In agreement with the previous finding that treatment of cells with piceatannol at 4°C could induce Cbl loss, these experiments also suggested that piceatannol and piceatannol-like compounds were directly reacting with Cbl in the absence of cellular degradation machinery, and in addition, suggested that the oxidation of piceatannol was sufficient for this reaction to occur.

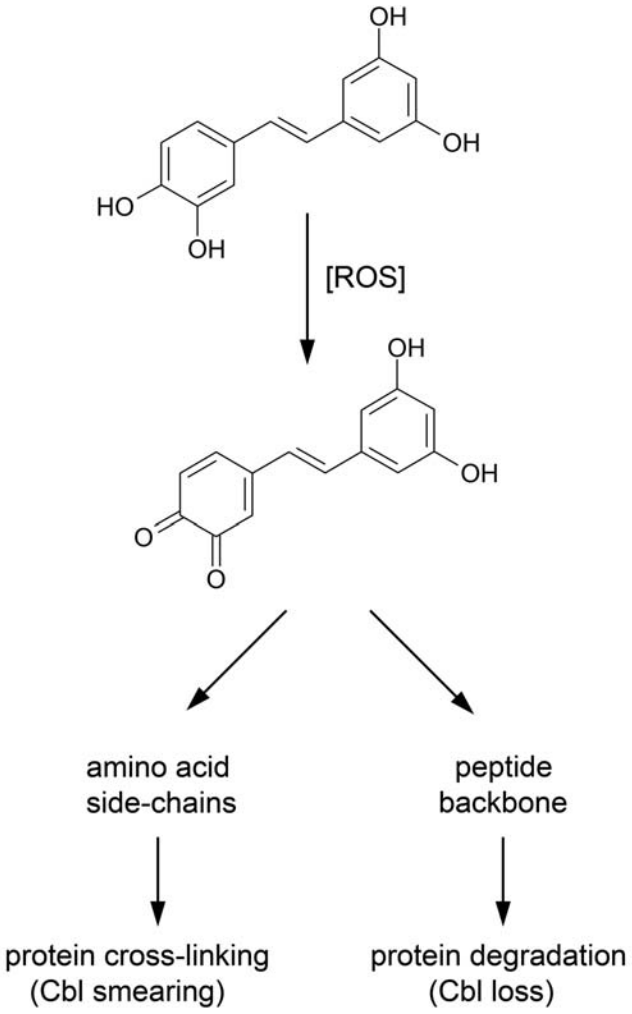


Figure 4.19 – Schematic of the new hypothesis for piceatannol-induced Cbl-loss.
Piceatannol becomes oxidized into an o-benzoquinones in the presence of reactive oxygen species (ROS). These o-benzoquinones can readily react with nucleophilic atoms from Cbl's amino acid side chains and promote Cbl cross-linking, visualized as smearing on overexposed Western blots of piceatannol treated cell lysates. The o-benzoquinones can also react with Cbl's peptide backbone, inducing scission of the polypeptide backbone and leading to the loss of Cbl observed after piceatannol treatment.

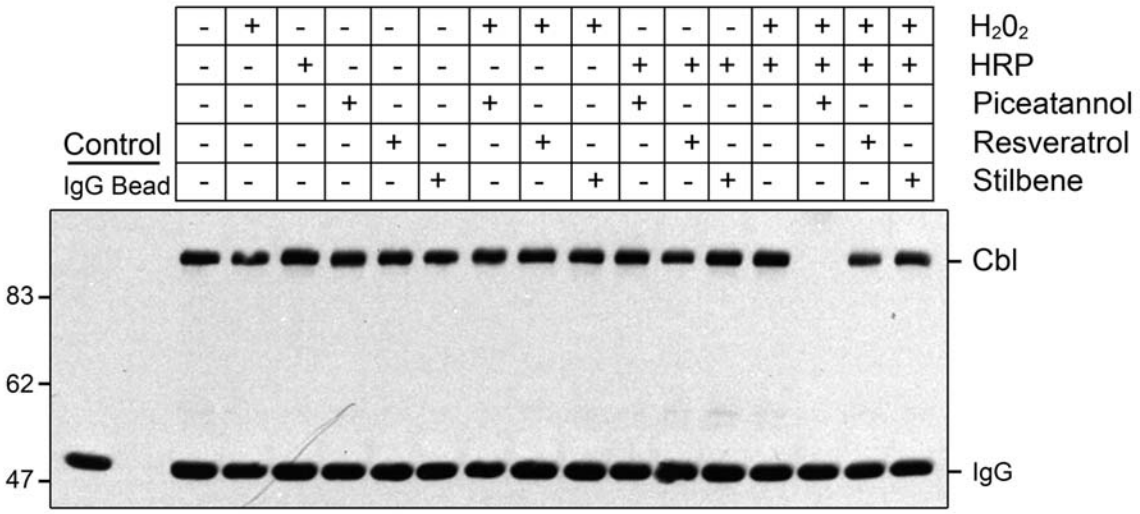


Figure 4.20 – Piceatannol induces the loss of Cbl *in vitro* in the presence of reactive oxygen species.

Cbl immunoprecipitations from 70Z/3 cells were incubated in PBS with combinations of 10μM H_2O_2, 20ng horseradish peroxidase (HRP), 200μM piceatannol, 200μM resveratrol, and 200μM stilbene for 15 minutes at room temperature. Western blots were probed for Cbl with the immunoprecipitating antibody. The IgG control represents an immunoprecipitation performed using lysis buffer instead of lysates. The Bead control represents an immunoprecipitation performed without antibody. The heavy-chain of the immunoprecipitating antibody is included at the bottom of the blot as a protein control demonstrating the specificity of the degradation. (Representative of n=3)

4.2.14 Oxidation is required for piceatannol-induced Cbl loss in vitro

To further examine the requirements for piceatannol oxidation in piceatannol-induced Cbl loss, I introduced N-acetyl cysteine to the *in vitro* assay conditions to neutralize ROS produced by the H_2O_2/HRP system. In the presence of this anti-oxidant, piceatannol-induced Cbl loss was dramatically decreased (Figure 4.21), further demonstrating the requirement for oxidation of piceatannol in this process.

4.2.15 To induce Cbl loss, piceatannol must be oxidized into an o-benzoquinone

To further demonstrate the requirement for the oxidation of piceatannol's catechol ring for piceatannol-induced Cbl loss, I examined the *in vitro* reactivity of pairs of compounds that possessed catechol ring structures with or without acetate ester groups protecting the hydroxyl groups. Compared to piceatannol as the positive control, none of the other compounds were as effective at reducing Cbl levels *in vitro* when combined with reactive oxygen species (Figure 4.22). Two sets of paired compounds, differing only by the presence of acetate ester protective groups on the ortho-hydroxyl groups, compounds 7 and 29 and compounds 25 and 24, both demonstrated that acetate esters present on compounds 7 and 25 prevented compound-induced Cbl loss *in vitro* compared to their respective unprotected control compounds, 29 and 24 (Figure 4.22).

To verify the involvement of the ortho-hydroxyl groups in the induction of Cbl loss by piceatannol-like compounds, I compared the ability of acetate ester protected compounds to induce Cbl loss *in vitro* after a pretreatment with porcine liver esterase. Compounds 7, 19, and 31 were unable to induce Cbl loss under normal conditions; however, after treatment with esterase, all three compounds induced a similar degree of Cbl loss (Figure 4.23). While the degree of Cbl loss was not as complete as that seen in the case of piceatannol, this difference in activity may be due to incomplete de-esterification of the compounds, resulting in reduced active concentrations compared with piceatannol. Overall, these results further demonstrated that oxidation of ortho-hydroxyl groups on a catechol-ring structure is required for compound-induced Cbl loss.

Of note, compound 24 and de-esterified compound 19 were able to induce modest Cbl loss *in vitro* whereas these compounds and the acetate ester analogue of compound 24, compound 25, did not demonstrate the ability to induce Cbl loss *in vivo* in the 70Z/3

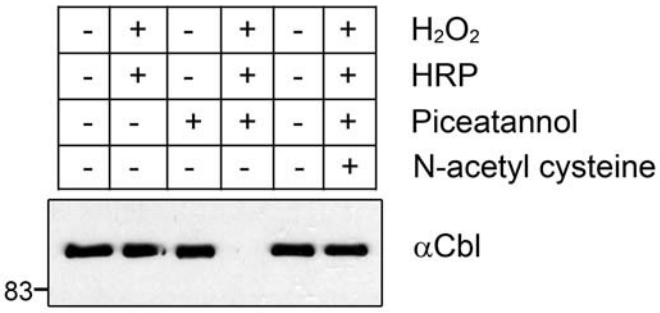


Figure 4.21 – Antioxidants inhibit piceatannol-induced Cbl loss *in vitro*.
Cbl immunoprecipitations from 70Z/3 cells were incubated in PBS with combinations of 10μM H_2O_2, 20ng horseradish peroxidase (HRP), 200μM piceatannol, and 1mM N-acetyl cysteine for 15 minutes at room temperature. Western blots were probed for Cbl with the immunoprecipitating antibody. (Representative of n=3)

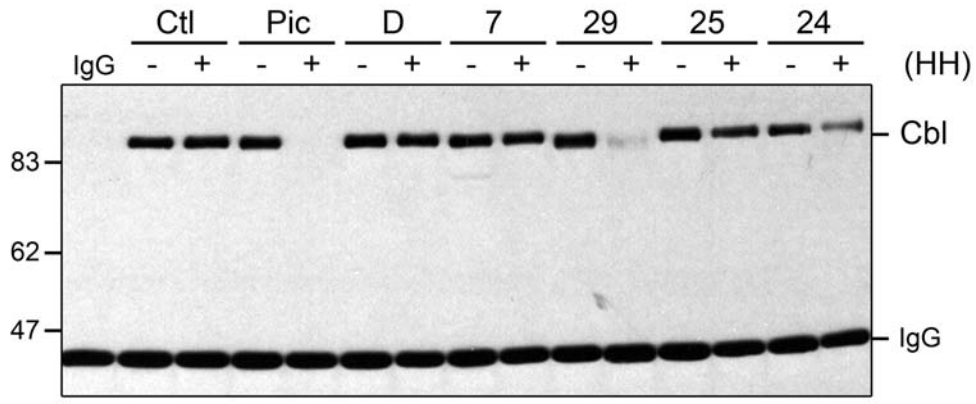


Figure 4.22 – Protection of the reactive ortho-hydroxyl groups in piceatannol-like compounds abrogates their ability to induce Cbl loss.

Cbl immunoprecipitations from 70Z/3 cells were incubated in PBS with or without 10μM H_2O_2 and 20ng horseradish peroxidase (HH), in the presence of nothing (Ctl), 200μM piceatannol (Pic), DMSO (D), 200μM compound 7 (7), 200μM compound 29 (29), 200μM compound 25 (25), or 200μM compound 24 (24) for 15 minutes at room temperature. The compounds in the compound pairs 7 and 29, as well as 25 and 24 are identical except for acetate ester groups protecting the ortho-hydroxyl substitutions on compounds 7 and 25. Western blots were probed for Cbl with the immunoprecipitating antibody. The IgG control (IgG) represents an immunoprecipitation performed using lysis buffer instead of cell lysate. The heavy-chain of the immunoprecipitating antibody is included at the bottom of the blot as a protein control demonstrating the specificity of the degradation. (Representative of n=2)

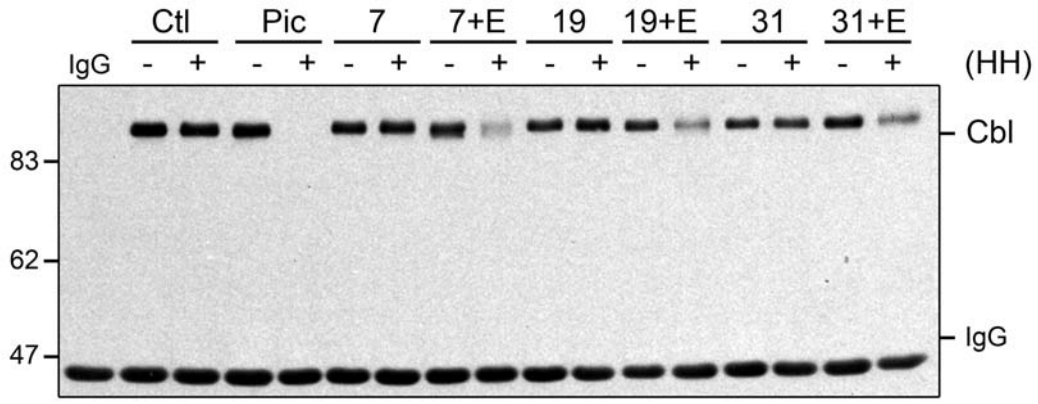


Figure 4.23 – Enzymatic removal of the acetate ester protective groups restores the ability of piceatannol-like compounds to induce Cbl-loss *in vitro*.

Cbl immunoprecipitations from 70Z/3 cells were incubated at room temperature for 15 minutes in PBS with or without 10μM H_2O_2 and 20ng horseradish peroxidase (HH), in the presence of nothing (Ctl), 200μM piceatannol (Pic), 200μM compound 7 (7), 200μM compound 7 pretreated with bovine esterase (7+E), 200μM compound 19 (19), 200μM compound 19 pretreated with bovine esterase (19+E), 200μM compound 31 (31), or 200μM compound 31 pretreated with bovine esterase (31+E). Western blots were probed for Cbl with the immunoprecipitating antibody. The IgG control (IgG) represents an immunoprecipitation performed using lysis buffer instead of cell lysate. The heavy-chain of the immunoprecipitating antibody is included at the bottom of the blot as a protein control demonstrating the specificity of the degradation. (Representative of n=2)

cells (Figure 4.23). This observation further confirmed the results of the *in vivo* compound screen that indicated that there are specific structural requirements for compound-induced Cbl loss *in vivo*.

4.2.16 Piceatannol is converted into an o-benzoquinone in the presence of ROS

To verify that piceatannol and piceatannol-related compounds were being converted into o-benzoquinones, I examined the spectral properties of these compounds in the presence and absence of ROS. O-benzoquinones absorb light at a wavelength of 400nm much more strongly than do catechols (Sugumaran et al., 1992), therefore by subtracting the absorbance at 400nm (A_{400}) of an aqueous solution of compound from the A_{400} of the compound solution in the presence of ROS, over time I will be able to determine if these compounds are becoming o-benzoquinones as this value, the ΔA_{400}, will increase. Aqueous solutions of piceatannol, compound 25, and compound 29 at concentrations of 500μM were prepared and their A_{400} was monitored every 5 seconds for 150 seconds. These values were subtracted from the comparable A_{400} readings from these compounds in the presence of ROS. The ROS in these solutions was provided by the addition of the potent oxidant $NaIO_4$ at a concentration of 10μM. All of these compounds demonstrated increasing ΔA_{400} values over time compared to the negative control resveratrol, indicating the formation of o-benzoquinones (Figure 4.24). Resveratrol was included as a negative control, as this compound is unable to form an o-benzoquinone due to the positions of its hydroxyl groups (Figure 4.1). In order to confirm the requirement for ROS in the conversion of the piceatannol-like compounds into o-benzoquinones, the same experiment was performed in the presence of N-acetylcysteine. The presence of this anti-oxidant completely abrogated the increasing ΔA_{400} over time, indicating that it blocked the formation of o-benzoquinones (Figure 4.24). These data demonstrated that o-benzoquinones were formed when the catechol containing compounds piceatannol, compound 25, and compound 29, were exposed to oxidant. This offers further proof that the oxidation of piceatannol-like compounds is sufficient to induce the loss of Cbl as all three of these compounds have been shown to induce Cbl loss *in vitro* (Figure 4.23).

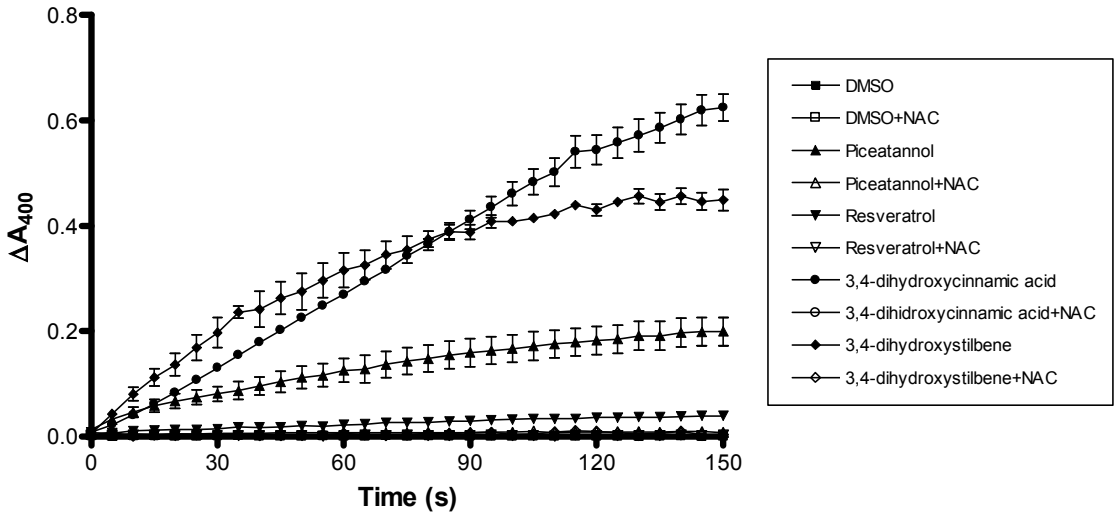


Figure 4.24 – Piceatannol reacts with ROS to form o-benzoquinones.

Aqueous solutions of piceatannol, resveratrol, compound 25 (3,4-dihydroxycinnamic acid), and compound 29 (3,4-dihydroxystilbene) at concentrations of 500µM were prepared and their A_{400} was monitored every 5 seconds for 150 seconds. ΔA_{400} values were generated by subtracting these values from the comparable A_{400} readings from these compounds in the presence of 10µM $NaIO_4$ as a source of ROS. ΔA_{400} values were plotted over time to identify the presence of o-benzoquinones, which absorb more strongly at 400nm. Resveratrol was included as a negative control, as this compound is unable to form an o-benzoquinone due to the positions of its hydroxyl groups. Compound 25 was included as a positive control, as this compound is known to generate o-benzoquinones when oxidized. In order to confirm the requirement for ROS in the conversion of the piceatannol-like compounds into o-benzoquinones, the same experiment was performed in the presence of 1mM N-acetyl cysteine. The presence of this anti-oxidant completely abrogated the increasing ΔA_{400} over time, indicating that it blocked the formation of o-benzoquinones. (Each data point represents the mean of n=3 ± SD)

4.2.17 Oxidation of unprotected catechols by metal ions leads to o-benzoquinone formation

As a further demonstration of the oxidative conversion of piceatannol and piceatannol-like compounds into o-benzoquinones, I examined piceatannol-induced DNA damage. Production of DNA single and double strand breaks by catechols has been extensively characterised and is known to require transition metal ions, which oxidize catechols to generate o-benzoquinones (Hirakawa et al., 2002). The metal ions are subsequently oxidized by atmospheric oxygen, generating H_2O_2 in the aqueous solution (Hirakawa et al., 2002; Subramanian et al., 2004). DNA strand breaks are induced by reduced metal-peroxide attack complexes produced as a result of catechol oxidation (Hirakawa et al., 2002; Subramanian et al., 2004). To confirm the formation of o-benzoquinones from the oxidation of unprotected piceatannol-like compounds, I examined the ability of piceatannol, and several piceatannol-like compounds, to induce DNA damage using an *in vitro* assay in which piceatannol or piceatannol-like compounds were incubated with supercoiled pSP72 plasmid DNA, in the presence or absence of $CuCl_2$. In the absence of $CuCl_2$, none of the compounds had any effect on the plasmid DNA. Similarly, in the absence of piceatannol or its related compounds, $CuCl_2$ alone was unable to induce DNA strand breaks (Figure 4.25). DNA single strand breaks were visualized on an agarose gel as the loss of the most rapidly migrating band, which is composed of supercoiled DNA (Figure 4.25). DNA double strand breaks were visualized as the collapse of all of the DNA bands and by the generation of a smear of DNA. Resveratrol, which does not contain a catechol group, was only able to induce DNA single strand breaks in the presence of $CuCl_2$ (Figure 4.25). Both piceatannol and compound 29, which contain unprotected catechols, were able to induce DNA double-strand breaks in the presence of $CuCl_2$ as an oxidizing agent (Figure 4.25). Compounds with partially or fully protected catechol groups that were unable to reduce the Cu^{2+} ions to Cu^{+} through conversion into o-benzoquinones, including compounds 7 and 10, were unable to induce any DNA damage (Figure 4.25). These data supported my supposition with respect to piceatannol that oxidation of unprotected catechol groups generated o-benzoquinones.

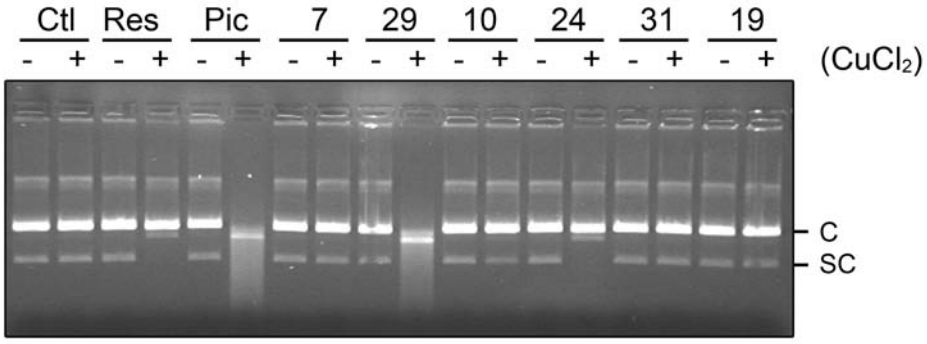


Figure 4.25 – Piceatannol-mediated DNA damage is enhanced in the presence of ROS.

Gel electrophoresis pattern of supercoiled plasmid DNA. *In vitro* reactions were made up in HBSS with or without 50µM $CuCl_2$ and contained 200ng of pSP72 plasmid and 50µM resveratrol (Res), piceatannol (Pic), compound 7 (7), compound 29 (29), compound 10 (10), compound 24 (24), compound 31 (31) or compound 19 (19). Reactions were incubated at 37°C for 1 hour, and visualized on a 1% agarose gel with ethidium bromide. Supercoiled plasmid DNA (SC) is relaxed by DNA single strange breaks into coiled DNA (C). DNA smearing indicates multiple double strand DNA breaks. (Representative of n=3)

4.2.18 Piceatannol-mediated tyrosine kinase inhibition is partially dependent on the oxidation of piceatannol into an o-benzoquinone

While copper ions are very proficient at generating DNA damage in the presence of hydroxystilbenes (Subramanian et al., 2004; Azmi et al., 2005), they are not the only transition metal ion capable of inducing chemical oxidation. When I combined piceatannol with an aqueous solution of $MnCl_2$, I observed the appearance of the characteristic red/brown colour consistent with the presence of oxidized piceatannol (data not shown) (Boekelheide et al., 1979; Boekelheide et al., 1980). Piceatannol's ability to inhibit tyrosine kinase activity was initially characterised in an *in vitro* kinase assay that used a buffer containing $MnCl_2$ (Geahlen and McLaughlin, 1989; Thakkar et al., 1993). Manganese ions are commonly included in tyrosine kinase assays as some kinases have been shown to be dependent upon this metal ion for their activity. It was therefore possible that piceatannol's ability to inhibit tyrosine kinase activity was not simply due to its ability to bind to tyrosine kinase substrate binding pockets (Geahlen and McLaughlin, 1989), but was also due to its oxidation into a chemically reactive o-benzoquinone that could react with and neutralize amino acids critical for kinase activity. To test this possibility and to understand how oxidized piceatannol reacts with proteins in general, I performed *in vitro* kinase assays using the lipid raft fractions of 70Z/3 and U937 cells as an enriched source of tyrosine kinase activity.

To determine if oxidation of piceatannol and piceatannol-like compounds was required for tyrosine kinase inhibition, I performed *in vitro* tyrosine kinase assays, using standard *in vitro* kinase assay buffers containing $MnCl_2$, with or without pretreatment with the antioxidant glutathione. The kinase assays, initiated by the addition of ATP, were stopped after 15 minutes with the addition of 4x Laemmli buffer containing β-mercaptoethanol and the resulting tyrosine kinase activity was visualized by Western blotting for phosphotyrosine. Piceatannol completely inhibited ATP-induced kinase activity, and this inhibition was partially blocked by pretreatment with glutathione (Figure 4.26). Kinase inhibition mediated by the Src-family kinase inhibitor PP1, used as a control, was not affected by the addition of glutathione. This result demonstrated that piceatannol's tyrosine kinase inhibitory activity was dependent upon oxidation of

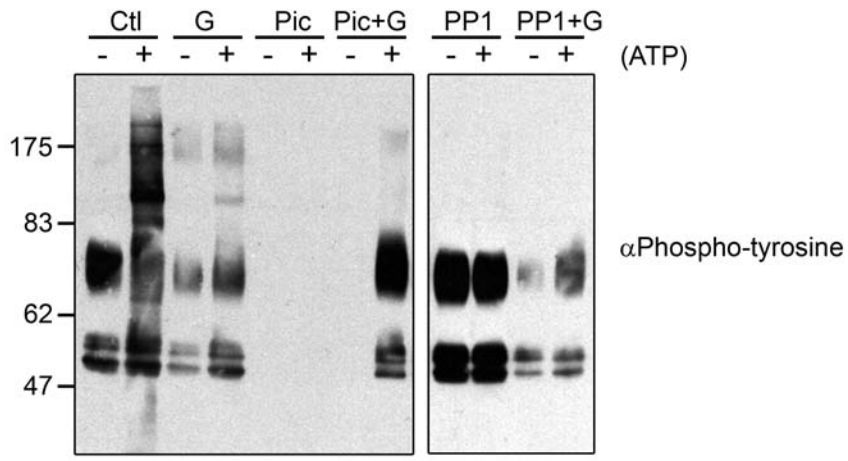


Figure 4.26 – Piceatannol-mediated kinase inhibition is inhibited by antioxidants. Western blots of *in vitro* kinase assays probed for phosphotyrosine. Isolated lipid rafts were resuspended in kinase assay buffer and were pretreated with DMSO (Ctl), 1mM glutathione (G), 200µM piceatannol (Pic), 200µM piceatannol and1mM glutathione (Pic+G), 10µM PP1, or 10µM PP1 and 1mM glutathione (PP1+G). After pretreatment, samples were diluted with kinase assay buffer with or without ATP (final concentration of 100µM ATP). After a 15 minute incubation at room temperature, kinase reactions were terminated by the addition of Laemmli buffer. (Representative of n=2)

piceatannol.

To further demonstrate that piceatannol's tyrosine kinase inhibitory activity was dependent upon oxidation, I resuspended lipid rafts in kinase assay buffer with or without $MnCl_2$. *In vitro* kinase assays, visualized by Western blotting for phosphotyrosine, indicated that the presence of $MnCl_2$ enhanced piceatannol's tyrosine kinase inhibitory activity (Figure 4.27). However, in the absence of $MnCl_2$, piceatannol was still able to inhibit tyrosine kinase activity, but to a lesser degree. This experiment confirmed the previous result with the caveat that piceatannol's kinase inhibition was only partially dependent upon catechol oxidation.

To further verify that piceatannol's tyrosine kinase inhibition was dependent upon its oxidation into an o-benzoquinone, I compared the ability of several piceatannol-like compounds to inhibit tyrosine kinase activity *in vitro* in the presence of $MnCl_2$. While compounds containing unprotected catechol groups were all able to inhibit tyrosine kinase activity to some degree, including compounds 29 and 25, piceatannol displayed the most complete inhibition (Figure 4.28). Compounds without catechol groups, including resveratrol, compound 6 and compound 8, or compounds with protected catechol groups, including compounds 7, 10 and 24, were unable to inhibit lipid raft associated tyrosine kinase activity *in vitro* (Figure 4.28). These data further confirmed that oxidation of catechol groups, regardless of overall chemical structure was sufficient for the inhibition of tyrosine kinase activity. This implies that o-benzoquinones, due to their electrophilic nature, were directly modifying nucleophilic amino acids within the tyrosine kinases and impeding their function (Novakovic et al., 2003). This result also demonstrated that piceatannol's unique chemical structure enhanced its ability to inhibit tyrosine kinase activity compared to other catechol containing compounds, presumably by enabling it to competitively inhibit substrate binding in the kinase binding pocket in addition to directly modifying key amino acids.

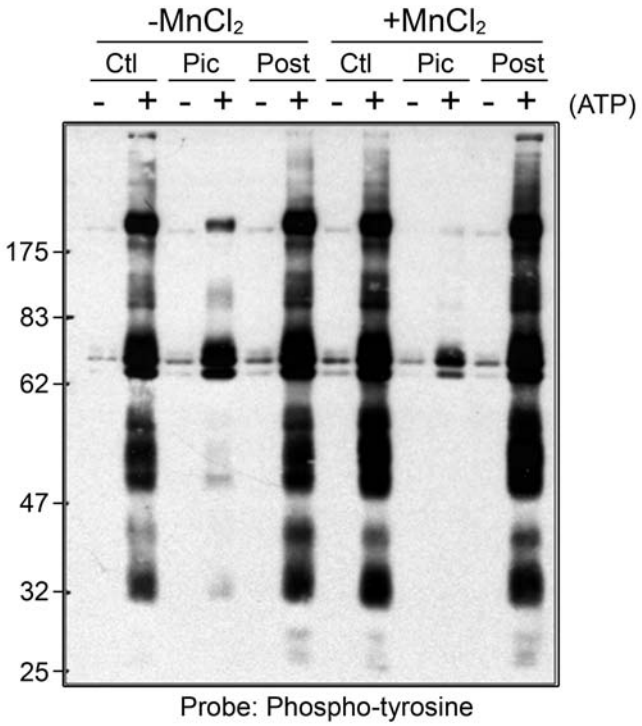


Figure 4.27 – Piceatannol-mediated kinase inhibition is enhanced in the presence of ROS.

Western blots of *in vitro* kinase assays probed for phosphotyrosine. Isolated lipid rafts were resuspended in kinase assay buffer with $MgCl_2$ (-$MnCl_2$) or with $MnCl_2$ (+$MnCl_2$) and were pretreated for 15 minutes on ice with DMSO (Ctl) or 200μM piceatannol (Pic). After pretreatment, samples were diluted with kinase assay buffer with or without ATP (final concentration of 100μM ATP). After a 15 minute incubation at room temperature, Ctl and Pic kinase reactions were terminated by the addition of Laemmli buffer, whereas Post samples were treated with 200μM piceatannol and incubated on ice for 15 more minutes prior to termination. (Representative of n=2)

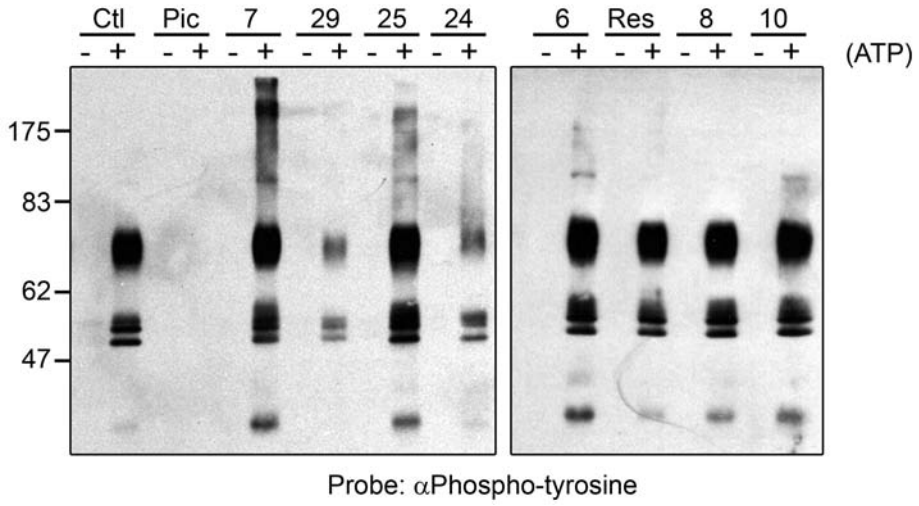


Figure 4.28 – Catechol-containing piceatannol-like compounds are not as efficient as piceatannol at inhibiting tyrosine kinase activity.

Western blots of *in vitro* kinase assays probed for phospho-tyrosine. Isolated lipid rafts were resuspended in kinase assay buffer and were pretreated with DMSO (Ctl), 200μM piceatannol (Pic), 200μM compound 7 (7), 200μM compound 29 (29), 200μM compound 25 (25), 200μM compound 24 (24), 200μM compound 6 (6), 200μM resveratrol (Res), 200μM compound 8 (8), or 200μM compound 10 (10). After pretreatment, samples were diluted with kinase assay buffer with or without ATP (final concentration of 100μM ATP). After a 15 minute incubation at room temperature, kinase reactions were terminated by the addition of Laemmli buffer. (Representative of n=2)

4.2.19 Piceatannol irreversibly inhibits matrix metalloproteinase-9 in the presence of ROS

Having observed that oxidized piceatannol directly modifies proteins, and knowing that piceatannol oxidation has the potential to reduce metal ions, I next examined this compound's ability to inhibit matrix metallo-proteases (MMPs). MMPs are a family of proteases that rely on a co-ordinated Zn^{2+} ion for their catalytic activity and also have a conserved methionine 1,4-turn adjacent to this catalytic zinc ion (Stocker et al., 1995). Both of these features could be susceptible to disruption by piceatannol, leading to MMP inhibition: the Zn^{2+} ion could be reduced in the process of oxidizing piceatannol (Hirakawa et al., 2002; Subramanian et al., 2004), and the conserved structural methionine could directly react with piceatannol's o-benzoquinone form due to the nucleophilic properties of the amino acid's sulphur atom (Ahlfors et al., 2003). In an *in vitro* reaction, I treated recombinant MMP9 with piceatannol alone, ROS alone, or both together. After 15 minutes, reactions were diluted with zymography loading buffer and MMP activity was then examined by gelatin zymography. While ROS alone mildly inhibited MMP9 activity, piceatannol was only able to inhibit MMP9 activity in the presence of ROS. As little as 10μM piceatannol, in the presence of ROS, was sufficient to completely inhibit MMP9 activity (Figure 4.29). Further confirming the requirement for oxidation of piceatannol in its inhibition of MMP9 activity, pre-treatment of MMP9 with N-acetyl cysteine prevented piceatannol/ROS inhibition of gelatinase activity (Figure 4.29). Since piceatannol treatment was performed prior to the gelatin zymography, these results demonstrated that oxidized piceatannol can irreversibly inhibit MMP9 activity. The irreversible nature of piceatannol's MMP9 inhibition suggests that this due to chemical modification or the reduction of the catalytic Zn^{2+} ion. The observation that piceatannol alone was not able to mediate MMP9 inhibition suggests that reduction of the Zn^{2+} ion is not involved, and implies that this inhibition is due to the chemical modification of MMP9 by oxidized piceatannol.

4.2.20 Piceatannol-induced protein loss has a broad specificity in vitro

Having established a model in which oxidized piceatannol directly reacted with and blocked the function of several different proteins in *in vitro* reactions; I no longer

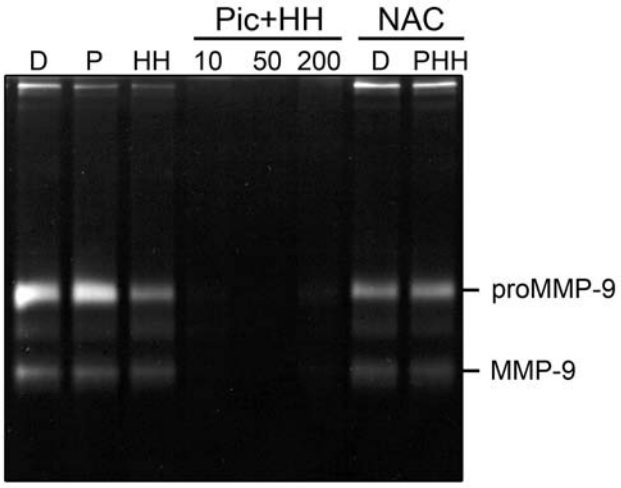


Figure 4.29 – Piceatannol-mediated MMP inhibition is enhanced in the presence of ROS.

Gelatin zymography of *in vitro* reactions with MMP-9. 10ng of recombinant MMP-9 was incubated in PBS with or without 2mM N-acetyl cysteine for 5 minutes on ice (NAC). DMSO (D), 200µM piceatannol (P), 100µM H_2O_2 and 0.02U of HRP (HH), 200µM piceatannol with 100µM H_2O_2 and 0.02U of HRP (PHH), or 100µM H_2O_2 and 0.02U of HRP with 10 µM, 50 µM or 200µM piceatannol (Pic+HH). After a 10 minute incubation at room temperature, the reactions were terminated by the addition of 4x Zymography loading buffer and run on a 12% SDS-PAGE gel embedded with 1mg/mL gelatin. (Representative of n=2)

believed that piceatannol was specifically acting upon Cbl proteins. This made sense considering that there are many different amino acids with nucleophilic atoms in their side chains, none of which are unique to Cbl, which could react with o-benzoquinones, including cysteine, methionine, histidine, tryptophan, serine, tyrosine, lysine, arginine, and proline (Ahlfors et al., 2003). To specifically test this hypothesis, I immunoprecipitated several different proteins, including c-Cbl, c-Src, Lyn, and PDGFRβ, and used them in *in vitro* reactions with piceatannol and/or ROS. Western blotting confirmed that similar to c-Cbl, all of the proteins were lost in the presence of piceatannol and ROS (Figure 4.30). These data indicated that, at least *in vitro*, piceatannol-induced protein-loss had a broad specificity.

4.2.21 Piceatannol induces the loss of specific proteins in vivo

Having determined that piceatannol was able to induce the loss of several proteins other than Cbl *in vitro*, I next tested to see if the loss of these same proteins occurred *in vivo*. 70Z/3 mouse B cells and wtCbl-overexpressing 3T3 mouse fibroblasts were treated for 1 hour and 3 hours, respectively, with increasing concentrations of piceatannol. NP40 detergent lysates of the treated cells were then examined by Western blotting for the presence of c-Cbl, Cbl-b, PDGFRβ, c-Abl, c-Src, Lyn, actin, and Grb2. In contrast to the broad specificity observed in the *in vitro* assays, piceatannol was only able to induce the loss of a small subset of proteins *in vivo*, including, c-Cbl, Cbl-b, PDGFRβ, and c-Abl (Figure 4.31 A and B). Lyn, c-Src, actin and Grb2 were unaffected by piceatannol treatment. These results demonstrated that *in vivo* piceatannol-induced protein-loss remained selective for certain types of proteins. In addition to the selective loss of proteins *in vivo*, Lyn shifted to a lower apparent molecular weight after piceatannol treatment (Figure 4.31 B). This molecular weight shift is reminiscent of a change in the tyrosine phosphorylation status of the protein, and was likely the result of piceatannol-induced kinase inhibition.

4.2.22 Piceatannol-induced loss of c-Cbl associated proteins may be c-Cbl dependent

All of the proteins I examined for sensitivity to piceatannol treatment, except actin, are known to directly associate with c-Cbl

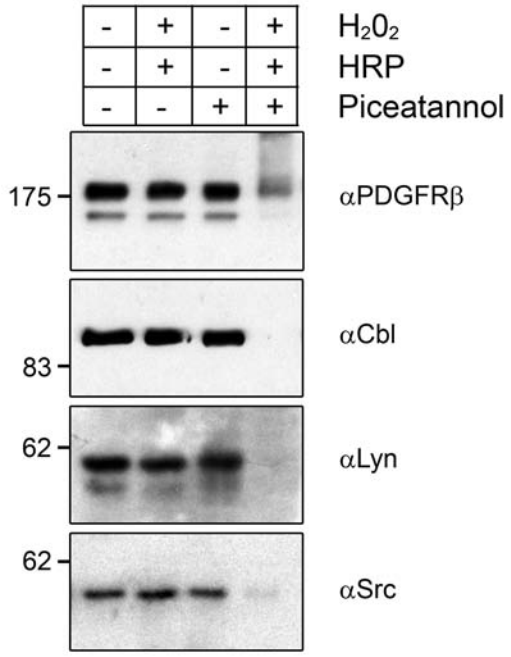


Figure 4.30 – Piceatannol induces the loss of other proteins *in vitro* in the presence of reactive oxygen species.

Immunoprecipitations of PDGFRβ, c-Cbl, Lyn, and Src were incubated in PBS with combinations of 10μM H_2O_2, 20ng horseradish peroxidase (HRP), and 50μM piceatannol for 15 minutes at room temperature. Western blots of these *in vitro* reactions were probed for with the specific immunoprecipitating antibodies. (Representative of n=2)

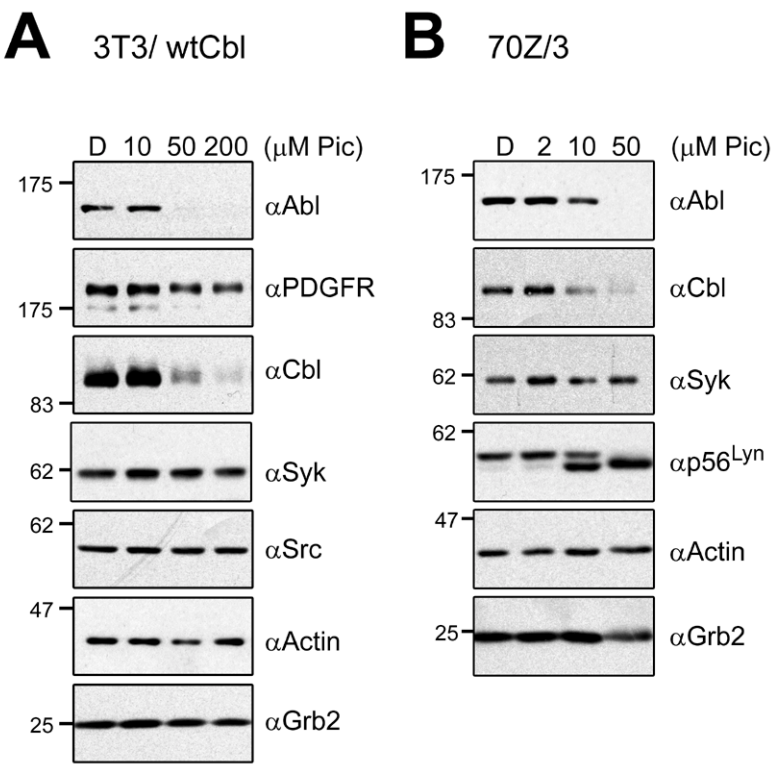


Figure 4.31 – Piceatannol induces the loss of specific Cbl-associated proteins.

A WtCbl overexpressing murine 3T3 fibroblasts and **B** 70Z/3 cells, were treated with DMSO alone (D) or increasing concentrations of piceatannol (10, 50, and 200μM) for 1 3, and 8 hours. Western blots of cell lysates were probed for Cbl, the Cbl interacting proteins c-Abl, PDGFRβ, Syk, Src (or the Src-family kinase Lyn) and Grb2, and the Cbl non-interacting protein actin. (Representative of n=3)

(Andoniou et al., 1994; Donovan et al., 1994; Odai et al., 1995; Panchamoorthy et al., 1996; Tezuka et al., 1996; Bonita et al., 1997; Lupher et al., 1998; Miyake et al., 1999; Sanjay et al., 2001). To rationalize the discrepancy between the *in vitro* and *in vivo* sensitivities of these various proteins, I was interested in determining if the Cbl-associated proteins that I found to be sensitive to piceatannol *in vivo* were sensitive due to their association with Cbl. I treated $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs with increasing doses of piceatannol for 3 hours and examined protein levels by Western blotting (Figure 4.32). Similar to the c-Cbl overexpressing mouse fibroblasts and the 70Z/3 cells (Figure 4.31), the amounts of c-Cbl, c-Abl and PDGFRβ were all decreased in the $Cbl^{+/+}$ MEFs following piceatannol treatment (Figure 4.32). However, in the $Cbl^{-/-}$ cells, none of the proteins examined were affected, indicating that c-Cbl may be involved in the piceatannol-induced loss of c-Cbl associated proteins *in vivo*.

4.2.23 Piceatannol induces the functional loss of receptor protein tyrosine kinases

Based on my observation that piceatannol treatment led to a loss of PDGFRβ, I was interested in determining if this loss was sufficient to affect how cells responded to PDGF-BB. To test this, I examined the MAPK activation status in $Cbl^{+/+}$ or $Cbl^{-/-}$ MEFs that were pre-treated with piceatannol for 8 hours or 10 minutes, prior to a 10 minute stimulation with PDGF-BB (Figure 4.33). Neither cell line was robustly affected after the 10 minute pretreatment, demonstrating that piceatannol's activity as a kinase inhibitor does not have a major impact upon PDGFRβ-induced MAPK activation. The 8 hour pretreatment with piceatannol prevented PDGF-BB induced ERK1/2 phosphorylation only in the $Cbl^{+/+}$ cells (Figure 4.33), demonstrating that the piceatannol-induced loss of PDGFRβ protein levels and function may be Cbl-dependent.

4.2.24 Piceatannol-induced loss of EGFR correlates with reduced EGFR signalling

To examine if piceatannol treatment could be used to induce the loss of Cbl-associated oncogenic receptors in a cancer cell model, I pretreated the A431 squamous cell carcinoma cell line, which overexpresses EGFR, with increasing concentrations of piceatannol prior to stimulation with EGF. Western blots of the A431 cell lysates indicated that at higher concentrations of piceatannol, both Cbl and EGFR protein levels are reduced and that this corresponds to reduced activation of MAPKs in response to

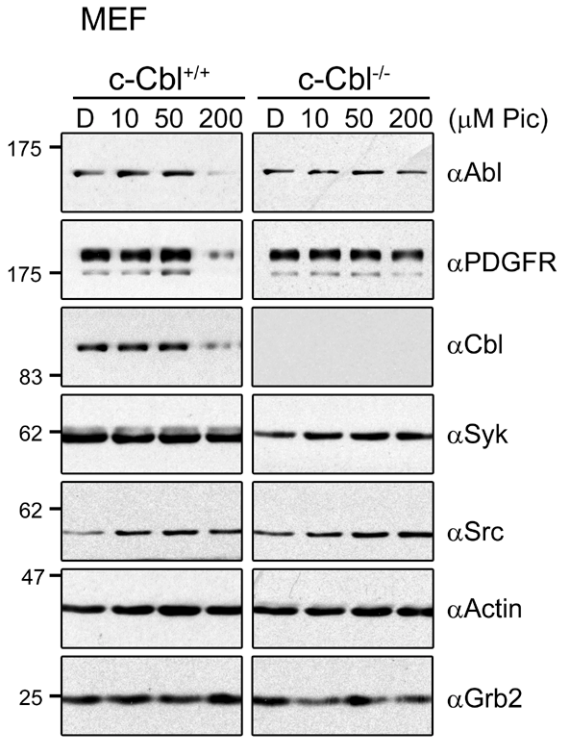


Figure 4.32 – Piceatannol-induces the loss of specific Cbl-associated proteins in a Cbl-dependent manner.

$Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs were treated with DMSO alone (D) or increasing concentrations of piceatannol (10, 50, and 200μM) for 3 hours. Western blots of cell lysates were probed for Cbl, the Cbl interacting proteins c-Abl, PDGFRβ, Syk, Src and Grb2, and the Cbl non-interacting protein actin. (Representative of n=3)

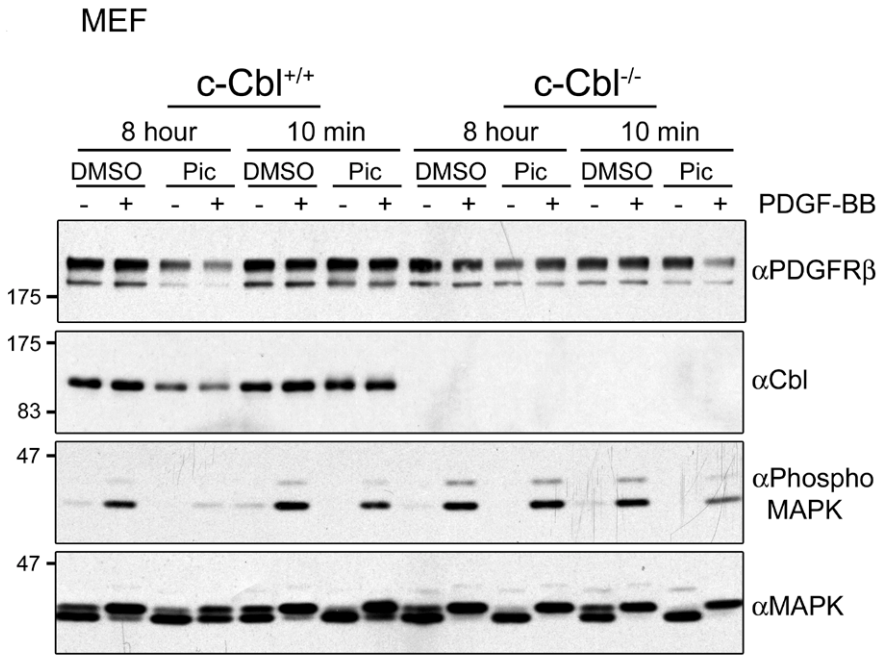


Figure 4.33 – Piceatannol induces Cbl-dependent functional loss of PDGFRβ.
$Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs were treated with DMSO or 200μM piceatannol for 8 hours or 10 minutes, followed by a 10 minute stimulation with 50ng/ml PDGF-BB. Western blots of cell lysates were probed for PDGFRβ, Cbl, phospho-MAPK, and total MAPK. After an 8 hour pretreatment with piceatannol, PDGFRβ levels are reduced only in the $Cbl^{+/+}$ MEFS, leading to a reduced ability to activate MAPK in response to PDGF-BB treatment. (Representative of n=5)

EGF stimulation (Figure 4.34). This result suggests that the piceatannol-induced loss of Cbl-associated signalling proteins may be beneficial as a treatment for a variety of cancers promoted by misregulated Cbl-associated PTKs.

4.2.25 γ-Radiation increases piceatannol-induced Cbl-loss

Radiation therapy is used to treat many different cancers. The principle behind this treatment is that radiation induces the formation of ROS within treated cells, which leads to ROS-mediated DNA damage and cell death. Having demonstrated that many of piceatannol's cellular effects were mediated by an oxidized form of piceatannol, and having demonstrated that piceatannol may be beneficial as a cancer treatment through its ability to induce the loss of Cbl-associated signalling proteins, I was next interested in examining whether radiation-induced production of ROS within cells would enhance piceatannol's effects. I tested this hypothesis by pretreating 70Z/3 cells with increasing concentrations of piceatannol for 30 minutes prior to irradiation. Western blot analysis demonstrated that the combination of piceatannol and radiation increased the loss of Cbl over the effect of either treatment alone (Figure 4.35). This result demonstrates that piceatannol-induced Cbl loss is enhanced by *ROS in vivo*, and suggests that piceatannol may be effective as a combination therapy with cancer therapeutics that produce ROS.

4.3 Discussion

While searching for inhibitors of Cbl-mediated transformation, I observed that piceatannol treatment of cells induced the loss of Cbl. The aim of this chapter was to determine the mechanism by which piceatannol induced Cbl loss to better evaluate its potential as a cancer therapeutic. I initially hypothesized that piceatannol stimulated proteolytic cellular processes that are known to regulate Cbl. Exhaustive testing of these cellular processes, including apoptosis, proteasomal degradation, and lysosomal degradation disproved this hypothesis. To generate a new hypothesis, I used an alternative approach to determine how piceatannol was inducing Cbl loss. I screened a small library of piceatannol-like compounds for their ability to induce Cbl loss on cell culture to determine which chemical structures were involved. Based on the common chemical structures identified in this screen and the observation that piceatannol treatment of cultured cells turned both the growth media and cell pellets brown, I

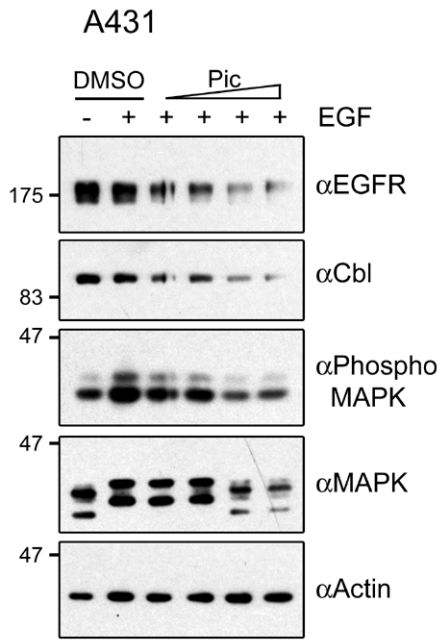


Figure 4.34 – The piceatannol-induced loss of RTKs correlates with reduced RTK signalling.

A431 cells were pretreated for 3 hours with increasing concentrations of piceatannol (32.5, 75, 150 and 300μM) prior to a 15 minute stimulation with 10ng/ml EGF. Western blots of cell lysates were probed for EGFR, Cbl, phospho-MAPK, and total MAPK. (Representative of n=3)

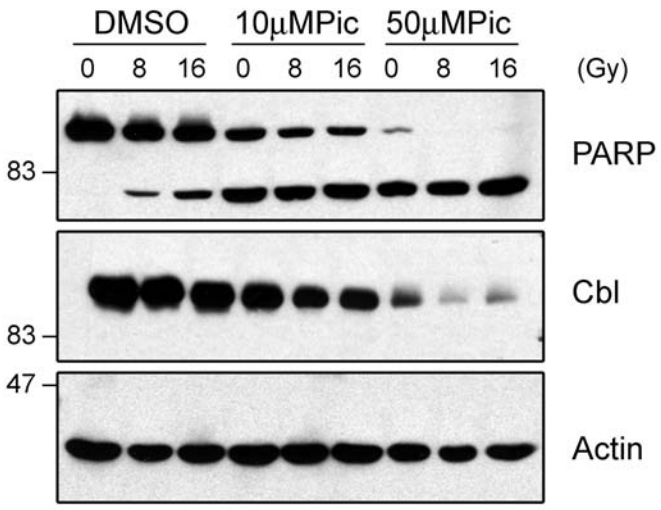


Figure 4.35 – γ-Radiation increases piceatannol-induced Cbl-loss.
Western blots of lysates from 70Z/3 cells pretreated for 30 minutes with DMSO, 10μM or 50μM piceatannol before receiving 0, 8, or 16 Gy of radiation. Cells were incubated for 8 hours at 37°C with 5% CO_2, prior to lysis. The top panel was probed for PARP, the middle panel was probed for Cbl, and the bottom panel was probed for actin. (Representative of n=1)

hypothesized that oxidation of piceatannol's catechol ring led to the formation of a chemically reactive o-benzoquinone that directly attacked Cbl, leading to the loss of c-Cbl and Cbl-b. To evaluate the merit of this hypothesis, I developed an *in vitro* reaction system that demonstrated that piceatannol oxidation induced the loss of immunoprecipitated Cbl. In addition, I showed that oxidation is involved in other properties associated with piceatannol, including its ability to inhibit tyrosine kinases and MMPs. This indicated that piceatannol was not as selective for Cbl as I had previously thought. I further examined this lack of selectivity and found that piceatannol treatment could also lead to the loss of a subset of Cbl-associated proteins *in vivo*, and that the loss of these proteins was Cbl-dependent. Finally, I demonstrated the potential for piceatannol to be used as a chemotherapeutic agent by demonstrating that piceatannol-induced loss of Cbl and Cbl-associated proteins correlates with reduced signalling from these proteins.

4.3.1 Piceatannol does not induce Cbl loss through the activation of cellular proteases known to regulate Cbl-protein levels

Cbl protein-levels are normally controlled by tightly regulated cellular processes, such as apoptosis (Widmann et al., 1998) and ubiquitin-mediated proteasomal and lysosomal degradation (Thien and Langdon, 2001; Yokouchi et al., 2001; Howlett and Robbins, 2002; Magnifico et al., 2003). I observed that prolonged treatment of cells with piceatannol was able to reduce the amount of Cbl detectable by Western blotting (Figure 4.2). I initially thought that piceatannol was mediating the apparent degradation of Cbl through the activation of one or more of these known Cbl-regulatory mechanisms. Ubiquitin-mediated degradation seemed likely to be involved based on the observed smearing of the Cbl band on Western blots (Figure 4.9, right panel). However, since piceatannol is also known to stimulate apoptosis (Wieder et al., 2001; Barton et al., 2004; Larrosa et al., 2004), I believed that this might also have been responsible for Cbl loss.

I failed to detect the presence of ubiquitin in Cbl immuno-precipitations, indicating that Cbl was not ubiquitinated in response to piceatannol treatment (Figure 4.9). Confirming the lack of involvement of ubiquitin-mediated degradation pathways, three different pharmacological inhibitors of the proteasome (Figure 4.10) and three

different pharmacological inhibitors of the lysosome (Figure 4.11) were unable to prevent piceatannol-induced Cbl loss.

I clearly ruled out the involvement of piceatannol-induced apoptosis in piceatannol-induced Cbl loss. I demonstrated that apoptosis, induced by means other than piceatannol treatment, was not sufficient to promote the loss of Cbl (Figure 4.7). This conflicts with the data of Widmann and colleagues, where the authors observed the degradation of c-Cbl upon the induction of apoptosis in Jurkat cells (Widmann et al., 1998). The loss of Cbl in these apoptotic cells was the indirect result of caspase activation and was concurrent with the generation of lower molecular weight c-Cbl protein degradation products (Widmann et al., 1998). It is unlikely that this mechanism of Cbl regulation is responsible for the piceatannol-induced loss of Cbl protein in this system for the following reasons: 1) the reduction in protein levels I observe upon treatment of cells with piceatannol is more dramatic than those observed by Widmann *et al.*(Widmann et al., 1998), 2) the loss of Cbl is not accompanied by the generation of low molecular weight Cbl degradation products, and is accompanied by an apparent increase in molecular weight of Cbl, which is visualized as a high molecular weight smear by Western blotting (Figure 4.9, right panel), 3) inhibition of caspase activity with a broad spectrum caspase inhibitor did not rescue piceatannol-induced Cbl loss (Figure 4.8).

As inhibition of the known Cbl-regulatory proteolytic pathways were unable to affect piceatannol-induced Cbl loss (Figures 4.8, 4.10, and 4.11), and I was unable to detect any changes in *Cbl* mRNA levels (Figure 4.5), I concluded that piceatannol causes Cbl loss at the protein level by a mechanism that has not previously been associated with Cbl.

4.3.2 Cbl loss is not the result of other properties ascribed to piceatannol

The base properties of piceatannol, the properties thought to be responsible for the many cellular effects associated with piceatannol treatment, include its ability to induce apoptosis, its ability to inhibit kinases, and its antioxidant properties. All of these properties, except for the antioxidant activity, are believed to involve piceatannol's modification of enzyme function through its association with these enzymes (Geahlen and McLaughlin, 1989; Thakkar et al., 1993; Law et al., 1999; Howitz et al., 2003; Seow

et al., 2004; Youn et al., 2005). Having ruled out the known cellular mechanisms involved in Cbl regulation as the causes of piceatannol-induced Cbl loss, I next examined which, if any, of piceatannol's known properties were able to mediate Cbl loss. By identifying which of piceatannol's properties was involved in Cbl loss, I hoped to be able to determine the enzyme(s) or cellular process responsible. Having already excluded apoptosis as the cause of piceatannol-induced Cbl loss (Figures 4.7 and 4.8); it remained to be determined whether kinase inhibition or antioxidant capacity was sufficient to induce Cbl loss.

I have demonstrated that piceatannol does not cause the loss of Cbl protein *via* its function as a kinase inhibitor. This is supported by the following data: 1) No other kinase inhibitor I tested was able to mimic the loss of c-Cbl induced by piceatannol, including the related phytochemical and broad spectrum tyrosine kinase inhibitor genistein, as well as the Src family kinase inhibitors PP1, PP2 and SU6656 (Figure 4.13). 2) The tyrosine phosphorylation status of c-Cbl does not affect its sensitivity to piceatannol (Figure 4.14). 3) The piceatannol-induced Cbl-dependent functional loss of PDGFRβ (Figure 4.33) occurred only after an extended pre-treatment with piceatannol, indicating that kinase inhibition, observable in B-cells after only 10 minutes of treatment (data not shown), is not directly involved. 4) The concentrations of piceatannol required for the induction of Cbl loss are more than fivefold greater than the concentrations of piceatannol commonly used to inhibit tyrosine kinases. These data show that piceatannol-induced Cbl loss is distinct from piceatannol's kinase inhibitory activity.

It is possible that piceatannol-induced Cbl loss is due to a combination of piceatannol's described properties; however, this is unlikely as resveratrol (Figure 4.16), epigallocatechin gallate and curcumin (Figure 4.15), have antioxidant, pro-apoptotic, and kinase inhibitory properties and none of these compounds has an effect on Cbl protein levels. It is interesting that resveratrol does not have any effect on Cbl levels, as not only does it possess many of the same properties as piceatannol (Zheng and Ramirez, 1999; Ashikawa et al., 2002; Gledhill and Walker, 2005; Ovesna et al., 2006), but it is almost structurally identical, differing by only one hydroxyl group (Figure 4.16). Thus it seems likely that this single hydroxyl group is sufficient to promote specific interactions

between piceatannol and the specific protein or proteins involved in the piceatannol-induced Cbl loss. While the possibility of resveratrol's conversion to piceatannol is intriguing (Potter et al., 2002), I did not determine whether the CYP1B enzyme was present in any of the cell lines tested and can thus only speculate that resveratrol's conversion to piceatannol is too slow or inefficient to attain the concentrations of piceatannol required to induce the loss of Cbl in these cells. What is clear is that the loss of Cbl is a property specific to piceatannol and not resveratrol, and that this property has heretofore been uncharacterized.

4.3.3 Chemical structures associated with Cbl loss reveal the mechanism of action

I was unable to define a cellular mechanism responsible for piceatannol-induced Cbl loss, and I was unable to associate Cbl loss with any of cellular processes affected by piceatannol's known properties. As a final attempt to determine if Cbl loss induced by piceatannol was due to piceatannol-mediated perturbation of a cellular process, I incubated piceatannol-treated cells at 4°C and examined Cbl levels by Western blotting. Incubation of cells with piceatannol at 4°C should have been sufficient to inhibit the majority of cellular enzymatic activity; however, it was not able to inhibit piceatannol-induced Cbl loss (Figure 4.12).

Having exhausted potential cellular and enzymatic processes that could have been responsible for piceatannol-induced Cbl loss; I decided to investigate the nature of the chemical properties that were required for piceatannol to induce Cbl loss. Because the chemical structure of a molecule determines its interactions and reactivity with its environment, I believed that this would provide insight into the mechanism of Cbl loss. I screened a library of piceatannol-like compounds for their ability to induce Cbl loss in cells to determine the chemical structures required for this process. Data from this screen supports a model in which piceatannol enters into cells and associates specifically with target proteins based on its small, rigid, planar structure. Subsequent oxidation of piceatannol generates o-benzoquinones, which readily react with nucleophilic atoms from Cbl's amino acid side chains (Ahlfors et al., 2003). This then leads to the cross-linking of Cbl, explaining the smearing observed by Western blotting after piceatannol treatment (Waidyanatha et al., 1998; Boatman et al., 2000). The o-benzoquinones can also react

with Cbl's peptide backbone, inducing scission of the polypeptide backbone and leading to the loss of Cbl observed after piceatannol treatment (Dean et al., 1997) (Figure 4.19). Several pieces of evidence provided by the screen, and later experimentally, support this model.

The most common structural feature of the screened compounds that correlated with the induction of Cbl loss *in vivo* was the presence of a catechol ring (Figure 4.18). Piceatannol and compounds 7, 29, and 31 all induced Cbl loss in the screen and all contain a catechol ring: unmodified in the case of piceatannol and compound 29, or protected with readily hydrolysable acetate esters in the case of compounds 7 and 31. Oxidation of catechols generates o-benzoquinones: eletrophilic compounds that are able to readily react with nucleophilic groups found in the side chains of many amino acids (Boatman et al., 2000). This observation supported the requirement for the oxidation of piceatannol to induce Cbl loss. Further corroborating the requirement for oxidation, catechol containing compounds in which the hydroxyl groups were modified by stable ether linkages, including compounds 9, 10, 13, and 18, were unable to mediate Cbl loss. Ether linkages, unlike ester linkages are resistant to hydrolysis and are thus not easily removed. Therefore ether-protected catechol rings are unable to undergo oxidation. The strongest evidence supporting the requirement for piceatannol oxidation comes from the development of an *in vitro* assay examining the minimal requirements for the induction of Cbl loss. In this assay immunoprecipitated Cbl is reacted in an aqueous solution in the presence of piceatannol with or without a source of ROS. Western blotting of the reactions indicated that piceatannol was only able to mediate Cbl loss in the presence of ROS, definitively demonstrating the requirement for oxidation of piceatannol in this process (Figure 4.20).

However, not all catechols or acetate ester-protected catechols were able to induce Cbl loss, providing evidence that there was some additional level of specificity to this reaction. Compound 12, the smallest catechol examined, was unable to induce Cbl loss, as was compound 15, a dihydro version of compound 7 (Figure 4.17). The common feature shared by these two compounds is reduced aromaticity compared with piceatannol or compound 7. Compound 12 has only the catechol ring and compound 15

electrically separates its two aromatic rings through the loss of the central double bond (Figure 4.17). Reduced aromaticity would also explain why several other catechol containing compounds were unable to induce Cbl loss, including (+)-catechin, gallic acid, and compounds 3, 23, 24, 25, 26, 27, 28, and 30. The requirement for aromaticity also strengthens the central involvement of oxidation in this process, as highly aromatic catechols that can support the loss of electrons that occurs during oxidation are more easily oxidized due to increased resonance stabilization of the resultant free electrons.

That protein selectivity is derived from the chemical structure of the piceatannol-like compounds is further implied by compound 33, which contains an o-benzoquinone structure. This compound was able to induce Cbl loss; however, it was also able to induce loss of actin, indicating that it lacked protein selectivity (Figure 4.18). As this compound is essentially a pre-oxidized catechol, this observation indicates that the location of catechol oxidation may provide the selectivity for the protein-loss reaction. The idea that the location of oxidation is important is also supported by the effectiveness of the acetate ester-protected compounds 7 and 31. These compounds require deprotection *via* esterases before they can be oxidized (Figure 4.23). I observed that compound 7, like piceatannol, was able to turn cell pellets a brown colour (data not shown), indicating that oxidized and therefore deprotected compound was present in the cells. However, unlike piceatannol, compound 7 did not change the colour of the media to a brown colour, implying that compound 7 is deprotected by intracellular esterases and that it is subsequently oxidized in the intracellular environment. While piceatannol can be oxidized outside of the cells, these observations imply that the protein selectivity I observe in cells treated with piceatannol-like compounds is dependent upon their intracellular oxidation. These observations indicate that only piceatannol-like compounds that can be effectively oxidized within cells are capable of inducing Cbl loss.

The importance of the ability to be efficiently oxidized in this phenomenon is also supported by the inability of compound 16, a cis-stilbene version of the trans-stilbene compound 7, to induce Cbl loss (Figure 4.18). A study of the differing anti-oxidant capacities of cis- and trans-resveratrol indicated that hydrogen abstraction is more thermodynamically favourable on trans- rather than cis-resveratrol and that this correlates

with the relative anti-oxidant capacities of these two molecules (Stivala et al., 2001). This suggests that compound 16's inability to induce Cbl loss may result from its reduced ability to become oxidized. There is also evidence to suggest that the physical difference in structure between compounds 7 and 16 is responsible for compound 16's inability to induce Cbl loss. The aromatic catechol containing compounds quercetin and compound 19, like compound 16, are also unable to induce Cbl loss in cells (Figure 4.18). These three molecules all differ slightly in structure from the Cbl loss-inducing compounds. The flavonol quercetin and the benzothiazole compound 19, contain extra ring structures compared to piceatannol and compound 7 (Figure 4.17). These differences should not significantly affect the oxidation potential of their respective catechol rings, as is suggested for compound 16. Therefore, the inability of these compounds, which have ring structures occupying areas where there would normally be empty space in a trans-stilbene, suggests that the shape of the cis-stilbene compound 16 would also interfere with compound-induced Cbl loss. Taken together, these observations indicate that there are specific structural requirements for the induction of Cbl loss *in vivo* beyond the requirement for catechol oxidation.

In addition to the requirement for chemical reactivity, provided by aromaticity-stabilized oxidation, the piceatannol-like compound screen supported the idea that there is also a requirement for structural specificity. This supports a mechanism for compound-induced Cbl loss in which compounds enter into cells, associate specifically with target binding partners based on their small, planar, rigid structure, where they are efficiently oxidized due to their aromaticity. The oxidized o-benzoquinones then readily react with nucleophilic atoms from Cbl's amino acid side chains and the peptide bonds in Cbl's backbone, inducing the smearing and loss of Cbl that I observe by Western blot (Figure 4.9, right panel).

4.3.4 Selectivity of piceatannol-induced protein-loss

In addition to inducing the loss of Cbl, piceatannol treatment of cells induced the selective loss of two Cbl-associated proteins. How does piceatannol's structure translate into the protein selectivity observed *in vivo* with respect to compound-induced protein-loss? The evidence from the compound screen suggests that the compounds are

associating specifically to some structure, protein or otherwise, within cells that has very restrictive binding requirements. However, the absence of a specific domain of Cbl that is responsible for its sensitivity to piceatannol and the lack of protein selectivity observed *in vitro* (Figure 4.2) rules out a direct association of the active piceatannol-like compounds with the target proteins, and suggests that the localization of proteins to areas within cells where these compounds are oxidized may determine specificity.

In vitro reactions combining several different immunoprecipitated proteins with piceatannol and ROS demonstrated that all of the proteins tested were sensitive to piceatannol-mediated protein-loss (Figure 4.29). These results indicated that the amino acid sequence and the tertiary structure of these proteins did not determine their sensitivity to the compounds. The only exceptions to this observation were the immunoprecipitating antibodies, whose levels were not noticeably affected by oxidized piceatannol. However, immunoglobulins may have evolved resistance, manifested by the selection of non-nucleophilic amino acids exposed at the surface of these proteins, due to the harsh extracellular and inflammatory environments in which they must function.

The lack of specificity evinced by the *in vitro* reactions agrees with the lack of specificity found in animal studies of benzene toxicity. Benzene toxicity is mediated by its oxidative metabolites, hydroquinones (Williams et al., 2002). Oxidation of hydroquinones leads to the generation of electrophilic benzoquinones that can form adducts with DNA, proteins and cellular antioxidants such as glutathione (Waidyanatha et al., 1998; Boatman et al., 2000; Williams et al., 2002; Galati and O'Brien, 2004). Protein and glutathione adducts, formed by the Michael-type addition of the sulfhydryl groups to the benzoquinone (Waidyanatha et al., 1998; Boatman et al., 2000; Williams et al., 2002), can lead to the formation of hydroquinones with up to 4 sulfhydryl groups (Waidyanatha et al., 1998; Boatman et al., 2000). Exposure of mice and rats to benzene leads to the accumulation of adducts of a number of proteins in many different tissues and leads to hepatotoxicity, nephrotoxicity and hematotoxicity (Boatman et al., 2000; Williams et al., 2002). Treatment of animals with high doses of other relatively non-specific electrophiles, such as nitric oxide, leads to neurotoxicity as these molecules react

with catalytic cysteine residues found on a number of proteins required for synaptic transmission (LoPachin and Barber, 2006).

In contrast to these toxic electrophilic compounds that lack *in vivo* specificity, daily exposure of mice to high doses of piceatannol did not lead to any toxic side effects and was partially protective towards artificially induced sepsis (Dang et al., 2004). This is reminiscent of the selectivity I observe in piceatannol-treated cells: not all catechols can mediate protein-loss (Figure 4.18 and 4.22) and only a subset of the proteins examined *in vitro* (Figure 4.30) retained their compound sensitivity *in vivo* (Figure 4.31). These data argue that the chemical structure of piceatannol and active piceatannol-like compounds imbue specificity not found in smaller catechol-containing compounds. It also suggests that *in vivo*, unlike the non-specific benzene-derived benzoquinones and other highly electrophilic toxins that can become readily oxidized in many different environments, that piceatannol-like compound oxidation occurs more readily in specific environments.

In contrast to the lack of selectivity for different proteins demonstrated in the *in vitro* reactions, the structural specificity of the piceatannol-like compounds implied by the screen and the *in vivo* studies would suggest a mechanism in which compounds bind specifically to target proteins, become oxidized, and then react with locally available nucleophilic protein components. The potential Cbl-dependence of PDGFRβ and c-Abl loss (Figures 4.32 and 4.33) also supports this idea, implying that the nature of their association with Cbl makes them more susceptible to attack from oxidized piceatannol than other known Cbl-associated proteins like Grb2, Src, and Syk. However, other pieces of data argue against this: 1) Syk and Src, proteins known to be functionally inhibited by piceatannol and thus assumed to physically associate with piceatannol (Geahlen and McLaughlin, 1989; Thakkar et al., 1993) are not affected by piceatannol-induced protein loss *in vivo* (Figures 4.31 and 4.32). 2) No specific region of Cbl was found to be resistant to piceatannol-induced loss *in vivo* (Figure 4.2). If piceatannol was binding to a region of Cbl, becoming oxidized and then attacking locally available nucleophiles I would expect that some parts of the Cbl protein would be resistant to piceatannol treatment as they would not be able to bind it efficiently or appropriately. In this scenario

the amino terminus, due to the high density of nucleophilic cysteine and methionine residues, would be particularly sensitive. However, v-Cbl, which incorporates only the amino-terminus of the c-Cbl protein, appears more piceatannol resistant than Δ1-355Cbl, which only incorporates the carboxy-terminus of the protein (Figure 4.2). The RING finger, due to the presence of Zn^{2+} ions, would also likely be involved in piceatannol sensitivity. However, p95Cbl, which lacks the RING finger, is just as susceptible as wtCbl (Figures 4.2 and 4.16).

The strength of the evidence against a direct interaction between piceatannol and its targets brings into question the Cbl-dependence of piceatannol-induced PDGFRβ and c-Abl protein-loss: does the process absolutely require Cbl, or are the two MEF cell lines sufficiently different due to the many uncontrollable variables involved in generating stable cell lines that they have a differential sensitivity to piceatannol. Upon closer examination, the cell lines are phenotypically different. Compared to the $Cbl^{-/-}$ cells, the $Cbl^{+/+}$ cells have a larger cytoplasm to nucleus ratio, are less uniform in shape, and grow more slowly (data not shown). Also, unlike the $Cbl^{-/-}$ cells, the $Cbl^{+/+}$ cells increase the viscosity of the culture medium after several days of growth (data not shown). A comparison of their piceatannol sensitivity demonstrates, however, that they are not differentially sensitive to piceatannol-induced cell death after 48 hours of treatment. The LC_{50} for $Cbl^{+/+}$ was 253 μM and the LC_{50} for $Cbl^{-/-}$ was 247 μM. This suggests that even though the cell lines are phenotypically different, this does not have an effect on their sensitivity to piceatannol. Considering these conflicting observations, I cannot discount the possibility that piceatannol-induced PDGFRβ and c-Abl loss is Cbl-dependent.

If this process is Cbl-dependent as my experiments have suggested, the question remains as to how piceatannol is inducing the selective loss of Cbl-associated proteins if it is not binding specifically to and oxidizing in the vicinity of Cbl? While I do not have experimental evidence to directly support it, the simplest explanation would involve Cbl helping to localize PDGFRβ and c-Abl to a specific area within cells that readily promotes the oxidation of piceatannol leading to the loss of these three proteins. This would explain the specificity in the structure of compounds able to induce protein-loss, as the structure of the compounds could make them more sensitive to oxidation under

specific environmental conditions that are found only in specific compartments within the cell. Or, the structure of the compounds could also make them more likely to be retained in specific compartments of the cell with more oxidative environments. It would also explain the Cbl-dependence and the protein selectivity in the absence of a specific interaction between the piceatannol-like compounds and the affected proteins, as the Cbl-dependent compartmentalization of these proteins would selectively expose them to oxidized piceatannol-like compounds. This scenario would rely upon an increased strength of interaction between Cbl and the affected tyrosine kinases compared to the unaffected kinases such as Src and Syk. This is possible as it is the kinase activity of many of Cbl's signalling partners, including the kinases listed above as well the EGFR (Figure 4.32), that mediates their strength of interaction with c-Cbl (Andoniou et al., 1994; Meisner and Czech, 1995; Bonita et al., 1997; Miyake et al., 1999). Thus the piceatannol-induced, Cbl-dependent loss of these Cbl-associated proteins may arise from their overactive kinase activity in transformed cell lines. The observation that Syk and Src were spared from piceatannol-induced loss may be due to piceatannol's ability to also inhibit their kinase activity and therefore reduce their affinity for Cbl (Geahlen and McLaughlin, 1989; Thakkar et al., 1993; Law et al., 1999). The fact that I did not see an appreciable loss of Grb2, which is involved in the association of c-Abl (Gaston et al., 2004), PDGFRβ (Bonita et al., 1997; Miyake et al., 1999), and EGFR (Meisner and Czech, 1995), with Cbl, may be due to the presence of comparatively higher levels of Grb2 in the cell lines examined.

While the mechanism behind the specificity of piceatannol-like compounds for Cbl, PDGFRβ and c-Abl is subject to further investigation, my data do not strongly support a model where these compounds mediate protein loss through direct binding. Instead, my data support an alternative model in which Cbl helps to localize strongly associated binding partners to areas within the cell where piceatannol-like compounds are more readily oxidized.

4.3.4.1 The NADP(H) oxidase hypothesis of piceatannol-induced Cbl loss.

One attractive hypothesis to explain location-specific piceatannol-induced protein-loss involves the local production of superoxide radicals by NADPH oxidases in

response to the activation of RTKs. This compartmentalized burst of ROS, required for effective RTK signalling (Lambeth, 2002; Chiarugi, 2005), does not impact the overall redox state in the cell (Halvey et al., 2005) and could therefore provide the protein selectivity I observe *in vivo*, compared with oxidized piceatannol's lack of selectivity *in vitro* (Figure 4.36).

NADPH oxidases from the Nox family of proteins are multi-pass transmembrane proteins that catalyze the transfer of electrons from $NADPH+H^+$ to O_2 to generate superoxide anions (Lambeth, 2002). The family, consisting of seven homologues, is thought to produce superoxide for several different purposes. The prototype member of the family, gp91phox, is expressed in phagocytic cells and is responsible for the respiratory burst used to kill invading pathogens (Lambeth, 2002). Other members of this family of proteins are involved in the production of ROS for signalling, oxygen sensing, and for specific biochemical reactions such as tyrosine iodination in the thyroid and osteoclast bone resorption (Lambeth, 2002). With respect to signal transduction, some Nox proteins have been shown to be associated with the plasma membrane where their activity is regulated in response to extra-cellular signals by the recruitment of a Rac-dependent protein complex (Lambeth, 2002; Finkel, 2003). These protein complexes all contain p22phox, which helps to recruit the adapter NOXO1 and its Rac-binding partner NOXA1 (Figure 4.36) (Bedard and Krause, 2007). NADPH oxidase complexes are also dependent upon tyrosine kinase activity to induce the local recruitment of signalling adapter proteins. Park *et al.* determined that for FcγRI induction of NOX, an inducible complex involving the FcγRI, Cbl, Grb2, and Shc is required (Park et al., 1999). Blocking the formation of this complex abrogated Nox activity. They also suggested that the Rac-GEF Vav, a Cbl-associated protein, is involved in this process (Park et al., 1999). The localization of all of these proteins to complexes proximal to the Nox-induced burst of ROS may make them sensitive to piceatannol-induced Cbl loss. While this work does not refer directly to RTK signalling, RTKs are thought to induce similar complexes and many of the same signalling proteins are thought to be involved, including Cbl, Grb2, Shc, Vav, and Rac (Ammendola et al., 2002).

I have observed the piceatannol-induced specific loss of many of the proteins

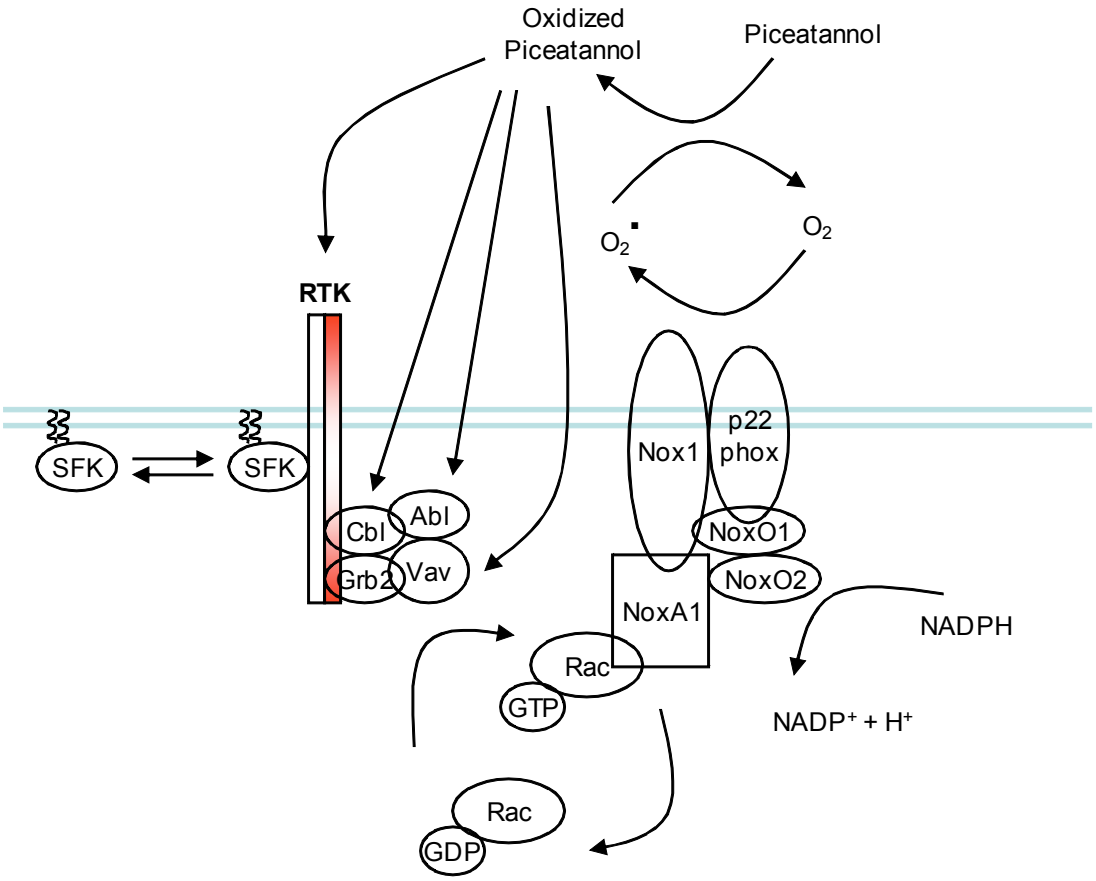


Figure 4.36 – Schematic of the NADP(H) oxidase hypothesis of piceatannol-induced protein loss.

Activated receptor tyrosine kinase (RTK) promote the activity of NADP(H) oxidase complexes through the recruitment of adaptor and effector proteins, such as Cbl and Vav, required for the activation of the small GTPase Rac. The NADP(H) oxidase complex transfers electrons from NADP(H) to O_2, generating superoxide radicals. Locally generated superoxide radicals are neutralized by piceatannol, through its oxidative conversion into an o-benzoquinone. Oxidized piceatannol attacks proteins that are firmly anchored in its local environment such as the RTKs, and some of the RTK associated signalling molecules such as Cbl, Vav, and c-Abl. Signalling molecules that are only transiently associated with the RTK complex, such as Src-family kinases (SFK), are not affected by oxidized piceatannol.

thought to be involved in Nox activation, including the Cbl-family proteins, Vav (Figure 5.5), and RTKs. I have not observed the loss of Grb2 in response to piceatannol treatment, however, as previously discussed; this may be due to comparatively higher concentrations of Grb2 present in the cell lines examined. I also do not see the loss of the Src-family kinases, implicated in the phosphorylation events leading to Nox complex formation; however, these proteins have not been shown to play important structural roles in the formation of these complexes (Park et al., 1999), and as such they may only transiently exist in the area proximal to the Nox protein complex and the Nox-induced burst of ROS. Conversely the piceatannol-induced loss of the NRTK c-Abl is likely due to its stronger affinity for c-Cbl (Gaston et al., 2004) and its critical role in promoting the appropriate localization of RTKs required for RTK-induced Nox activation (Ushio-Fukai et al., 2005).

4.3.5 Piceatannol as a cancer therapeutic

Concomitant with the piceatannol-induced loss of Cbl-associated PTKs, I observed an inhibition of signalling pathways associated with these proteins; specifically, the piceatannol-induced Cbl-dependent loss of PDGFRβ-mediated MAPK activation (Figure 4.34) and the piceatannol-induced loss of EGFR-mediated MAPK activation (Figure 4.33). These results provide support for revisiting piceatannol for use as an anti-cancer agent since many cancers are caused and/or aggravated by overactive kinase activity, and many of these kinases, such as Bcr-Abl, ErbB2, EGFR, and PDGFR are also Cbl-associated (Schmidt and Dikic, 2005). In addition, the inhibition of multiple PTKs, which may be possible through the use of piceatannol as a therapeutic, has proven to be a more effective cancer treatment than the inhibition of single PTKs (Bergers et al., 2003; Wong et al., 2004; Maione et al., 2006). I also demonstrate the potential for piceatannol to be used in combination with existing cancer therapies. As I had found that many of piceatannol's properties relied on the oxidation of piceatannol, including Cbl loss, kinase inhibition, and MMP inhibition, I hypothesized that cancer therapeutics known to induce ROS should enhance piceatannol's cellular effects. Treatment of cells with ROS-inducing γ-radiation enhanced piceatannol's ability to induce Cbl loss and cell death (Figure 4.35). As many cancer therapeutics are known to induce ROS (Davis et al.,

2001; Hideshima and Anderson, 2002; Ling et al., 2003; Engel and Evens, 2006; Ge et al., 2006; Baysan et al., 2007) and most cancer cells produce higher levels of ROS than normal cells (Loo, 2003), this further demonstrates piceatannol's potential to be used as a cancer therapeutic.

4.3.6 Summary

I have identified a novel property of piceatannol, whereby it is able to induce the loss of the c-Cbl proto-oncogene, two naturally occurring transforming c-Cbl mutants, and specific c-Cbl-associated PTKs. This loss was not dependent upon the cellular mechanisms known to regulate these proteins, and was not mediated by piceatannol's most well known and extensively characterized properties. Screening a library of piceatannol-like compounds, I found that the destruction of Cbl was not unique to piceatannol and could be mediated by small, rigid, aromatic compounds containing a catechol ring structure, which could be oxidized into a reactive o-benzoquinone. The oxidation of these compounds was sufficient for them to induce Cbl loss *in vitro* and the reactivity of these compounds towards nucleophilic amino acids also explained the smearing observed with piceatannol treatment *in vivo*. Oxidation was also shown to be necessary for piceatannol-induced tyrosine kinase inhibition and MMP9 inhibition, implying that the oxidation process may be a common mechanism unifying many of piceatannol's diverse functions. The specificity for the proteins affected in cells by piceatannol was determined to be independent of protein composition or structure and I hypothesize that it is due to the sub-cellular localization of the affected proteins. Importantly, the piceatannol-induced loss of RTKs led to a decrease in the ability of cells to promote MAPK activation in response to their stimulation with growth factor ligand. This data, coupled with the observation that ROS-inducing cancer treatments, such as γ-radiation, enhance piceatannol's cellular effects, supports the potential for piceatannol to be used as a broad spectrum, anti-cancer adjuvant therapy.

Chapter Five: The Application of Piceatannol-induced Cbl-loss to the Treatment of Multiple Myeloma

5.1 Introduction

5.1.1 Multiple myeloma

Multiple Myeloma (MM) is a clonal disorder of terminally differentiated B cells, known as plasma cells. This disease is characterized by an excess of monotypic plasma cells diffusely distributed throughout the blood stream and at multiple intramedullary sites. This proliferation of monotypic plasma cells is accompanied by an excess of monoclonal antibody in the blood and urine. MM usually begins as a premalignant tumour referred to as monoclonal gammopathy of undetermined significance (MGUS) (Kuehl and Bergsagel, 2002). MGUS is differentiated from MM by an intramedullary tumour content of less than 10%. This benign tumour occurs in 1% of adults over the age of 25 and progresses to MM at a rate of about 1% per year (Kuehl and Bergsagel, 2002). Stable disease with greater than 10% intramedullary content but without any bone-associated complications is referred to as smouldering myeloma. Similar to MGUS, smouldering myeloma is thought to be a premalignant tumour on the path towards the development of multiple myeloma (Kuehl and Bergsagel, 2002).

Primary genetic defects that allow for the development of MM are thought to be the result of errors in one or more of the B-cell-specific DNA modification processes involved in the production of the antibody repertoire, such as IgH class switching. Errors in IgH class switching, responsible for the development of the majority of MM cases (Kuehl and Bergsagel, 2002), can lead to the inappropriate rearrangement of the genome in locations other than the heavy chain gene locus. In most cases these aberrant translocations lead to the juxtaposition of proto-oncogenes to immunoglobulin transcriptional enhancers, leading to the inappropriate expression of these genes. These genetic translocations affect a number of genes simultaneously and thereby can potentially activate many distinct proto-oncogenes (Kuehl and Bergsagel, 2002). This makes MM distinct from most other types of cancer, which generally require more than one genetic alteration to induce cellular transformation. Secondary mutations lead to the progression MM, which is marked by an increase in the intramedullary tumour content,

and is associated with several bone and bone marrow related pathologies; including, osteolytic bone lesions, hypercalcaemia and renal failure, as well as anaemia and immunodeficiency (Kuehl and Bergsagel, 2002).

Similar to normal plasma cells, MGUS and MM cells depend upon the bone marrow microenvironment for growth and survival. In the most aggressive form of MM, know as plasma cell leukemia, myeloma cells become independent of the bone marrow microenvironment. As a result, this stage of MM is associated with extramedullary tumours in the blood, pleural fluid and skin, and is associated with a very poor prognosis (Kuehl and Bergsagel, 2002).

5.1.2 Multiple myeloma and the bone marrow microenvironment

Due to the accumulation of MM cells in the bone marrow, many normal bone functions are altered in MM including bone maintenance and haematopoietic cell development (Hideshima and Anderson, 2002; Kuehl and Bergsagel, 2002). In the bone marrow microenvironment, MM cells disrupt the normal balance of bone maintenance between bone absorbing osteoclasts and bone building osteoblasts. This disruption, over time, leads to reduced bone density at these intramedullary sites (Kuehl and Bergsagel, 2002). MM-associated osteolytic bone disease is characterized by the presence of bone pain and pathologic fractures, severely reducing quality of life, and is a major cause of MM-associated morbidity (Hideshima and Anderson, 2002). Osteolytic bone disease is also associated with the release of bone-associated calcium into the blood, leading to hypercalcaemia and kidney damage (Kuehl and Bergsagel, 2002).

The presence of an increased number of plasma cells in the bone marrow microenvironment, from approximately 4% of cells in healthy individuals to over 10% in diseased individuals, also leads to haematological symptoms. The increased burden of tumour cells makes it more difficult for normal blood cells, including red blood cells, neutrophils, and platelets, to mature and survive. This leads to complications including anaemia, thrombocytopenia, and immunodeficiency (Kuehl and Bergsagel, 2002).

Interactions between MM cells and the bone marrow stroma are also responsible for the phenomenon of cell-adhesion-mediated drug resistance (CAM-DR) (Hideshima and Anderson, 2002). CAM-DR is characterised by the resistance of tumour cells to the

induction of cell death pathways from many, mechanistically distinct cytotoxic agents (Hazlehurst and Dalton, 2001; Dalton, 2003). In MM cells this process is mediated by two separate effects linked to the activation of cellular adhesion molecules. First, integrin-mediated adhesion between MM cells and bone marrow ECM induces cell cycle arrest. While cell cycle arrest is not normally associated with promoting cancer proliferation, it significantly increases the resistance of MM cells to DNA damaging drugs (Hazlehurst and Dalton, 2001). Second, integrin-mediated interactions between bone marrow stromal cells and MM cells stimulate both cell types to secrete a number of growth factors and cytokines. Bone marrow stromal cells are stimulated to secrete IL-6 and IGF-1, while MM cells secrete TNF, IL-6, and VEGF (Dalton, 2003). These cytokines and growth factors lead to the activation of a number of signalling pathways associated with cell survival, including, NFκB, JAK/STAT, PI3K/AKT, and ERK1/2 (Dalton, 2003; Yasui et al., 2006). The importance of these adhesion-induced cyto-protective effects is emphasized by studies demonstrating that disrupting adhesion prior to drug treatment reverses CAM-DR (Hazlehurst et al., 2000). This study also suggests that disrupting MM adhesion and MM homing to the bone marrow niche may be a therapeutically viable option to treat this cancer (Hazlehurst et al., 2000; Hazlehurst and Dalton, 2001).

5.1.3 CXCR4

B cell homing to the bone marrow niche is most profoundly regulated by the chemokine CXCL12, also known as SDF-1α, which signals through the chemokine receptor, CXCR4. Bone marrow stromal cells abundantly secrete CXCL12, creating a CXCL12 gradient that is responsible for the chemoattraction of CXCR4-expressing plasma cells to bone marrow niches and is also responsible for their adhesion to bone marrow stromal cells (Moser et al., 2006) (Figure 5.1.). MM cells, like normal plasma cells, express CXCR4 and home to the bone marrow via this bone marrow stromal cells produced CXCL12 gradient.

CXCR4 is a G-protein coupled receptor, and like most chemokine receptors, signalling through this receptor is mediated by the G_i subfamily of heterotrimeric G-proteins. Ligand activated receptors trigger $G_{\alpha i}$ subunits to exchange GDP for GTP. This

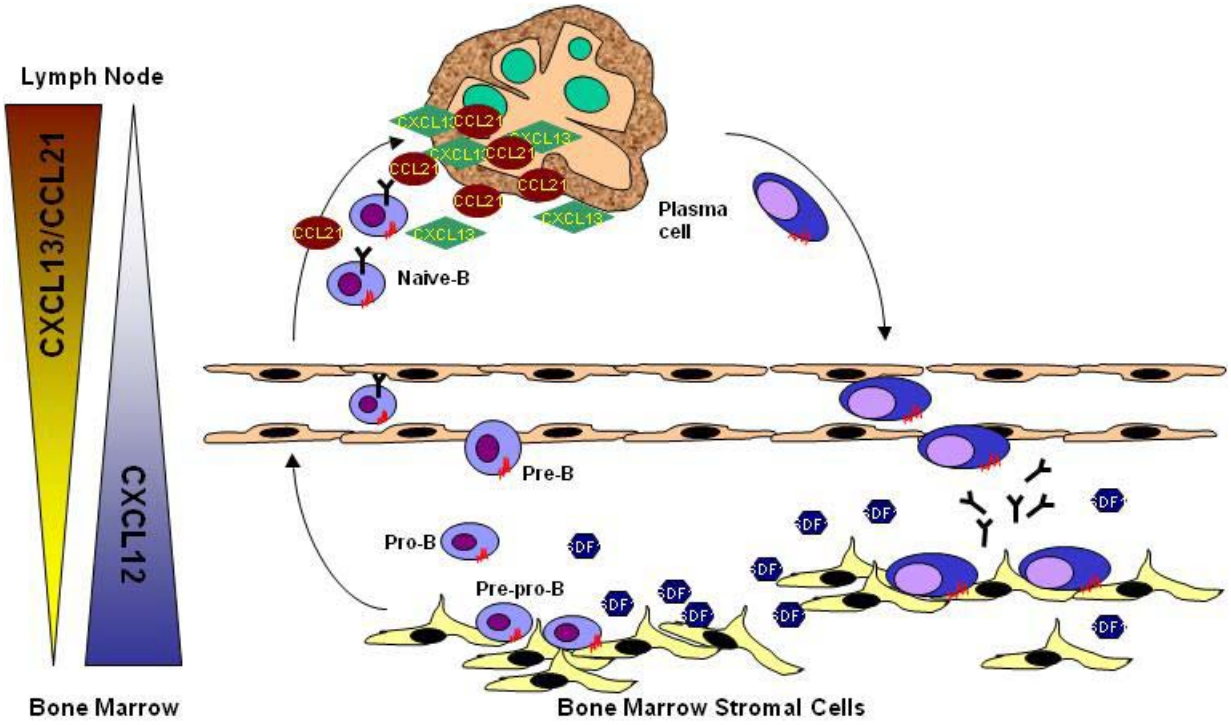


Figure 5.1 – The involvement of CXCL12 and CXCR4 in plasma cell maturation. Schematic summarizing the trafficking of B cells from the early stages of immature pre-pro B cells, to germinal center B cells and back to the bone marrow as mature plasma cells. CXCL13 and CCL21 gradients regulate homing and retention of immature B cells in the lymph nodes, whereas, a CXCL12 (SDF-1α) gradient regulates the homing and retention of mature plasma cells in the bone marrow. Multiple myeloma is a plasma cell cancer and multiple myeloma cells express CXCR4 and respond to CXCL12 similar to plasma cells. This results in the homing and retention of multiple myeloma cells in the CXCL12 producing bone marrow niche.

leads to the dissociation of the $G_{\alpha i}$ subunit from its associated $G_{\beta\gamma}$ heterodimer, and both components can then activate downstream effectors (Figure 5.2). While there are several effector proteins that have been shown to be recruited and activated as a result of CXCL12-mediated activation of CXCR4, only a few key signalling molecules have been demonstrated to be essential for the CXCL12-induced migration and adhesion phenotypes. These key molecules include the Src-family tyrosine kinases, the multifunctional adaptors c-Cbl and Cbl-b, the lipid kinase phosphotidylinositol-3-kinase (PI3K), the Rac-GEF Vav, and the small GTPase Rac (Okabe et al., 2005; Vicente-Manzanares et al., 2005; Okabe et al., 2006; Ortolano et al., 2006) (Figure 5.2.).

As suggested by the diverse assortment of proteins shown to be indispensable for CXCR4-mediated migration, stimulation of the CXCR4 receptor leads to the activation of a number of complementary signalling pathways that are all required for productive chemotaxis (Roland et al., 2003). One of the most prominent signals generated by CXCR4 ligation is the release of intra-cellular Ca^{2+} stores mediated by the activation of phospholipase C-β (PLCβ) (Bach et al., 2007). This process has been shown to be essential for CXCL12-mediated chemotactic responses in T cells; however, the nature of the requirement for this process is poorly understood (Bach et al., 2007). The specific involvement of Ca^{2+} mobilization in B cells, and specifically in plasma cells and MM, is even less well characterized than in T cells (Brandes et al., 2000). As well, most of the studies involving CXCL12-induced B cell migration have focused on $CD19^{+}$ B cells (Brandes et al., 2000; Palmesino et al., 2006), and since MM cells are generally $CD19^{-}$ (Bataille et al., 2006), their results may not be applicable to CXCL12-induced migration on MM cells .

Receptor ligation also induces the serine/threonine phosphorylation of CXCR4 by G-protein coupled receptor kinase (GRK). These phosphorylation sites, primarily located in CXCR4's C-terminus, are important for the recruitment of β-arrestins, a family of regulatory adaptor proteins. β-arrestins are important as both negative and positive regulators of chemokine receptor signalling. The binding of β-arrestins to GPCRs sterically inhibits G protein coupling, terminating active receptor signalling (DeWire et al., 2007; Moore et al., 2007). β-arrestins also promote receptor internalization by acting

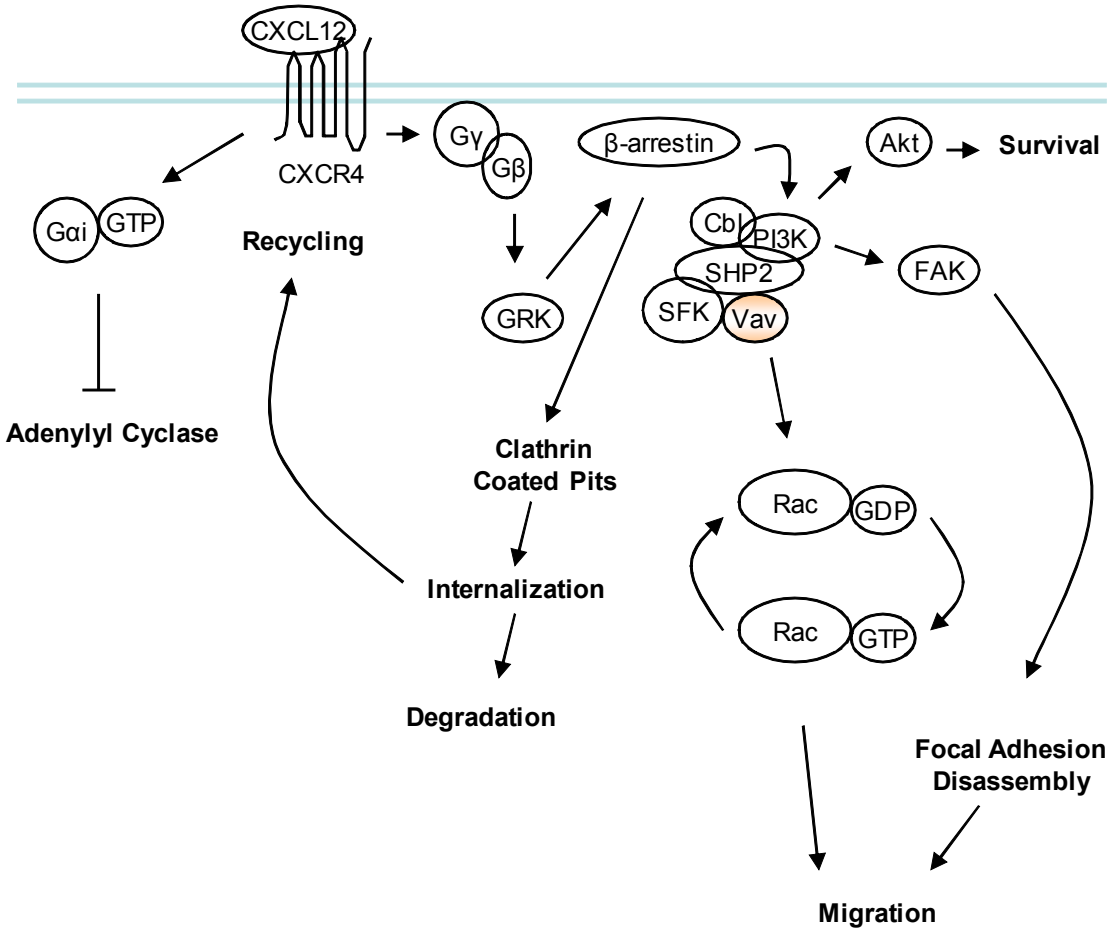


Figure 5.2 – Schematic representation of CXCR4 signalling pathways.

Binding of CXCL12 to CXCR4 activates the guanine nucleotide exchange activity of CXCR4, leading to the dissociation of $G_{\alpha i}$ from the G_{β} and $G\gamma$ G-protein subunits. The G_{β}-G_{γ} complex leads to the activation of GRKs, which phosphorylate the carboxy-terminus of CXCR4. β-arrestin binds to phosphorylated CXCR4 and recruits signaling effector molecules through protein-protein interactions. The Cbl family of proteins is indispensable in the nucleation of a β-arrestin-associated multi-protein signalling complex that promotes cell migration and cell survival. This multi-protein signaling complex is composed of proteins that have all been shown to associate with Cbl; including, PI3K, SHP2, Src family kinases (SFK), and Vav. The recruitment of β-arrestin to CXCR4 also leads to signal termination, receptor internalization, and degradation.

as an adaptor for several components involved in clathrin coated pit formation and function, including AP2 and clathrin (DeWire et al., 2007). On the other hand, β-arrestins are able to promote GPCR signalling by acting as multiprotein signalling scaffolds and recruiting a number of adaptor and effector molecules to the vicinity of the chemokine receptor (DeWire et al., 2007). SFKs and PI3K have been shown to associate with β-arrestins, nucleating signalling events required for the reorganization of the actin cytoskeleton; including, the activation of MAPKs and Rho-family GTPases, and the turnover of focal adhesions (DeFea, 2007). The Cbl-family of proteins has also been implicated in CXCR4-mediated migration (Chernock et al., 2001; Fernandis et al., 2004; Okabe et al., 2006) and are known to associate with both SFKs and PI3K (Schmidt and Dikic, 2005). This suggests that a multiprotein signalling complex, which is nucleated by β-arrestin and contains Cbl, SFKs and PI3K, is required for CXCR4-mediated migration (Figure 5.2).

5.1.4 The role of Cbl in CXCR4-mediated cell migration

The Cbl family of multifunctional adaptor proteins, including both c-Cbl and Cbl-b, act as positive regulators of many receptors and signalling effector molecules. Upon activation of a receptor, Cbl proteins are recruited from the cytosol to the activated receptor complex where they recruit signalling effector proteins including Src family kinases, Vav, and the p85 sub-unit of PI3K (Thien and Langdon, 2001). Cbl recruitment has been extensively studied in the context of RTKs (Thien and Langdon, 2005), however, little is known of its involvement in the transduction of signals from GPCRs. Recent evidence supports a role for Cbl in the formation of a multiprotein complex composed of Cbl, SHP2, and PI3K, required for CXCR4-mediated chemotaxis (Fernandis et al., 2004). This confirms earlier work that demonstrated an essential role for the formation of a protein complex containing Cbl, SHP2, PI3K, and Fyn (Chernock et al., 2001). The discovery that siRNA knockdown of either c-Cbl or Cbl-b was sufficient to inhibit CXCL12-induced T cell chemotaxis emphasizes the importance of such a signalling complex in the migration process (Okabe et al., 2006).

Cbl-family members also act as E3-ubiquitin ligases and mediate the ubiquitination of many of their binding partners; therefore, Cbl may also be important in

the negative regulation of the multiprotein complex that seems to be required for CXCR4-mediated chemotaxis. However, this is unlikely as the overexpression of wtCbl as well as the E3-ligase null 70ZCbl had the same stimulatory effect on CXCL12-induced migration (Fernandis et al., 2004). Only the TKB-defective G306E mutant of Cbl was able to decrease cell migration, emphasizing that Cbl's essential role in CXCR4-mediated migration is as an adaptor protein (Fernandis et al., 2004).

5.1.5 Current treatments for multiple myeloma

Traditional therapies for MM include chemotherapy, stem-cell transplantation and glucocorticoids. These treatments result in median survival times of 3-5 years (Kuehl and Bergsagel, 2002). Chemotherapeutic agents, primarily the alkylating agent melphalan, are most effective when used at myeloablative doses in combination with autologous stem cell support (Kuehl and Bergsagel, 2002). However, the intensive nature of this therapeutic regimen limits its use to younger and healthier patients. Steroids such as dexamethosone and prednisone are also used and these treatments act as non-specific anti-inflammatory agents targeting cytokine- and chemokine-induced survival signals required for MM maintenance and growth (Kuehl and Bergsagel, 2002). Neither of these therapies is curative and relapse inevitably occurs. The failure of these therapies is evinced by the fact that the five year survival rate of approximately 30% has changed little since 1980 (Kuehl and Bergsagel, 2002).

Based on the failings of the older MM therapeutics, recent strategies to develop effective MM therapies hinges upon simultaneously targeting the growth and survival pathways of these cancer cells, as well the protective interactions between MM cells with the bone marrow stromal environment (Harousseau et al., 2004). Several new chemotherapeutics have been developed to target both the MM cells and their interactions with the bone marrow microenvironment. These therapeutics are currently being tested for their safety and efficacy or are already being used clinically. These new treatments, including; arsenic trioxide, thalidomide derivatives, and bortezomib, have all been demonstrated to induce MM apoptosis, inhibit MM adhesion, prevent the release of growth factors and cytokines by bone marrow stromal cells, and reduce CAM-DR.

Arsenic compounds have been used to treat hematologic disorders since the 19th century (Berenson and Yeh, 2006). As_2O_3 mediates its cytotoxic effect in MM cells by promoting the activation of caspase-9 mediated apoptosis through the induction of ROS and oxidative stress in the mitochondria (Hideshima and Anderson, 2002; Baysan et al., 2007). It also sensitizes cells to other chemotherapies by downregulating anti-apoptotic proteins (Wetzler et al., 2006), inhibiting JAK/STAT signalling (Wetzler et al., 2006) and preventing the activation of NF-κB (Yu et al., 2007). Inhibition of these signalling pathways also inhibits the autocrine and paracrine signalling induced by MM association with bone marrow stromal cells responsible for increased growth, survival, and drug resistance of MM cells (Hideshima and Anderson, 2002). In preliminary Phase I/II clinical trials, As_2O_3 monotherapy induced stabilization and remission in patients with refractory or relapsed MM (Hussein, 2001). Based on *in vitro* studies with dexamethasone and ascorbic acid (Hayashi et al., 2002), As_2O_3 has also been evaluated in combination with these compounds. In a Phase II trial it demonstrated safety with few major adverse events and it demonstrated efficacy by increasing the median progression free survival by nearly twofold (Abou-Jawde et al., 2006). The increased sensitivity of cells treated with ascorbic acid, which reduces intracellular glutathione levels, suggests that these effects are largely mediated through the generation of ROS (Amadori et al., 2005; Engel and Evens, 2006).

Similar to As_2O_3, thalidomide and its derivatives, also induce mitochondrial oxidative stress leading to increased levels of ROS, disruption of mitochondrial membrane potential, and cell death (Ge et al., 2006). Disruption of cellular redox homeostasis is also linked to thalidomide-induced inhibition of NF-κB, MAPK, and PKC (Hideshima and Anderson, 2002). Loss of these signalling pathways prevents many of the protective effects associated with the bone marrow niche, including, adhesion, cytokine and growth factor production, drug resistance, and growth (Hideshima and Anderson, 2002). Thalidomide has been approved as first line therapy for newly diagnosed MM patients in combination with dexamethasone (Strobeck, 2007). Phase III trial data evaluating this combination determined that patients had a 63% response rate, compared to a 41% response rate with dexamethasone alone. Lenalidomide, a

thalidomide derivative has also shown promise as both a monotherapy (Richardson et al., 2002) and in combination with dexamethasone (Strobeck, 2007).

Bortezomib, a member of a new class of peptide boronate proteasome inhibitors, was recently approved by the FDA for use in patients with relapsed or refractory MM (Strobeck, 2007). This drug mediates its effects through the inhibition of the 26S proteasome. It is thought that the majority of bortezomib's effects are derived from its inhibition NF-κB activation (Cavo, 2006; Zavrski et al., 2007). Upon the inhibition of proteasome activity, the negative regulator of NF-κB, IκBα, is stabilized and prevents the translocation of NF-κB into the nucleus. Inhibition of NF-κB reduces a number of MM-beneficial effects derived from the association of the MM cells with the bone marrow niche; including, cytokine secretion, upregulation of adhesion molecules, and resistance to apoptosis (Cavo, 2006; Zavrski et al., 2007). Bortezomib also induces apoptosis through a less well characterized mechanism involving the generation of ROS in the mitochondria (Ling et al., 2003).

In addition to harnessing ROS to inhibit bone marrow niche-associated drug resistance and proliferative stimuli, newer therapies are being developed to target CXCR4-mediated migration and prevent MM from entering into the bone marrow niche (Hideshima and Anderson, 2002; Alsayed et al., 2006). While some of these therapies directly target the CXCR4 receptor (Alsayed et al., 2006), there are numerous other targets within the CXCR4 signalling pathway, including the Cbl-family of adaptor proteins.

5.1.6 Piceatannol as a treatment for multiple myeloma

The importance of c-Cbl and Cbl-b in CXCR4-mediated migration identifies them as potential targets for MM therapy. Knockdown of these two proteins has been shown to abrogate CXCR4-mediated migration (Okabe et al., 2006), which is responsible for CAM-DR and MM-associated lytic bone disease (Hideshima and Anderson, 2002; Kuehl and Bergsagel, 2002). I have discovered that piceatannol and several piceatannol-like compounds can induce the loss of both c-Cbl and Cbl-b. Others have demonstrated that piceatannol can induce apoptosis in cancer cells (Wieder et al., 2001; Barton et al., 2004; Larrosa et al., 2004), can inhibit a number of kinases important in CAM-DR (Law et al.,

1999; Hazlehurst and Dalton, 2001), and can inhibit the activation of NF-κB (Ashikawa et al., 2002; Dang et al., 2004; Youn et al., 2005). The potential for piceatannol to target multiple aspects of MM growth and survival, similar to many of the newer MM therapeutics, makes it a good candidate for use as a MM therapeutic. In addition, I have demonstrated that many of the effects attributed to piceatannol rely on the oxidation of this molecule. This presents an opportunity for synergy between piceatannol and many of the current MM chemotherapies, which are known to produce ROS (Ling et al., 2003; Ge et al., 2006; Baysan et al., 2007). In all, the weight of evidence supports studying of the feasibility of using piceatannol as an MM chemotherapy.

5.1.7 Objectives

I demonstrated the mechanism through which piceatannol and related compounds mediate many of their cellular effects in the Chapter Four. This mechanism, based on the oxidation of piceatannol's catechol ring, highlighted the potential usefulness of these compounds as cancer therapeutics. In this chapter I assess the feasibility of using piceatannol as a cancer therapeutic by demonstrating its effectiveness in a cancer model system. Screening a panel of human cancer cell lines, I identified cancer cells of haematopoietic origin as being particularly susceptible to piceatannol-induced cell death. Based on my knowledge of piceatannol's mechanisms of action I chose multiple myeloma cells as the cancer model system in which to test piceatannol's effectiveness as a cancer therapeutic. I propose that piceatannol has several therapeutic advantages in treating these cells: they are sensitive to piceatannol-induced cell death, they use Cbl-mediated signalling to promote disease associated morbidity and chemo-resistance (Hideshima and Anderson, 2002; Okabe et al., 2005; Vicente-Manzanares et al., 2005; Okabe et al., 2006), and there is a potential for synergistic interactions between piceatannol and several current MM therapeutics known to generate ROS (Ling et al., 2003; Ge et al., 2006; Baysan et al., 2007). I demonstrate the feasibility of this treatment in an *in vitro* model system of multiple myeloma by examining the selectivity of piceatannol for cancer cells, by examining the effect of piceatannol on several of the proteins involved in CXCR4-mediated cell migration, and by correlating the levels of

these proteins with piceatannol's ability to inhibit CXCL12-induced, CXCR4-mediated cell migration.

5.2 Results

5.2.1 Screening cancer cell lines for sensitivity to piceatannol-like compounds

Based on the findings in Chapter Four, which indicated that piceatannol may be useful as a cancer chemotherapy, I screened a panel of human cancer cell lines to assess their susceptibility to piceatannol and piceatannol-like compound-induced cell death. I hypothesized that the results of this screen would enable us to determine which cancer model system would be most advantageous to pursue with respect to using piceatannol as a therapy. I treated cells in 96 well dishes with increasing concentrations of piceatannol-like compounds for 48 hours before determining their viability by performing an MTT assay for adherent cells or by using trypan blue exclusion for non-adherent cells. From this screen I found that cancer cells of hematopoietic origin were the most sensitive to piceatannol-like compound-induced cell death (Tables 5.1 and 5.2). Of these cancer cell lines, I chose to study multiple myeloma cell lines for the following reasons: 1) these cells are sensitive to piceatannol-like compound-induced cell death (Table 5.2), 2) these cells use Cbl-mediated signalling pathways to exacerbate disease-associated morbidity and to promote resistance to chemotherapy (Okabe et al., 2005; Vicente-Manzanares et al., 2005; Okabe et al., 2006), and 3) Several current therapeutics used to treat multiple myeloma produce ROS (Ling et al., 2003; Ge et al., 2006; Baysan et al., 2007), allowing for possible synergy with piceatannol.

5.2.2 Piceatannol exhibits a large therapeutic index for multiple myeloma treatment

Many of the experiments I performed using piceatannol required relatively high doses to observe any potentially therapeutic effects, namely loss of Cbl-protein and apoptosis. I determined the therapeutic index for piceatannol to evaluate whether this might pose an issue for the use of piceatannol and related compounds as cancer treatments. MM cells reside in the bone marrow niche for the majority of their lifespan, therefore, to determine the therapeutic index I chose to compare the LC_{50} of primary normal human bone marrow stromal cells to the LC_{50} of the MM cell lines. The stromal cells proved to be exceptionally resistant to piceatannol-induced cell death (Figure 5.3). I

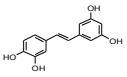
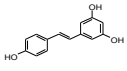
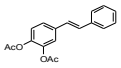
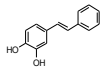
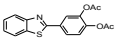
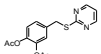
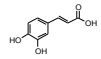
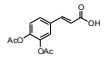
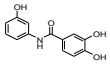
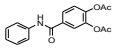
		Breast				SCC	Glioblastoma		NB	FS	Cervical	Colon
	Compound	HTB 26	HTB 126	HTB 129	HTB 132	A431	U87	U251N	LAN-1	HT1080	HeLa	HCT116
	Piceatannol	118.4	>500	467.1	461.1	487.2	>500	107.3	>500	>500	>500	465.4
	Resveratrol	145.1	43.1	125.0	123.2	47.9	167.5	120.9	97.9	44.2	144.4	40.0
	7	157.1	207.4	203.0	152.1	349.3	250.0	208.5	162.3	189.1	304.6	185.3
	29	71.5	175.9	98.0	80.4	90.1	99.0	140.9	72.7	89.3	170.8	171.9
	19	100.5	95.3	102.8	31.8	117.6	150.8	144.8	43.8	134.9	35.8	49.3
	31	206.0	390.1	361.3	304.8	373.8	379.9	376.2	230.8	330.9	374.6	417.7
	24	>500	>500	>500	>500	>500	>500	>500	>500	>500	446.9	362.2
	25	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	361.9
	3	nd	nd	nd	nd	353.6	409.3	200.0	186.4	nd	nd	nd
	23	nd	nd	nd	nd	>500	>500	>500	459.8	nd	nd	nd

Table 5.1 – LC_{50} values for piceatannol-like compounds in adherent human cancer cell lines.

Human breast cancer, squamous cell carcinoma (SCC), glioblastoma, neuroblastoma (NB), fibrosarcoma (FS), cervical cancer, and colon cancer cells were treated for 48 hours with increasing concentrations of piceatannol-like compounds. Cell viability was determined by MTT assay, and normalized to untreated controls. LC_{50} values (μM) were calculated mathematically as described in Chapter 2. Note: nd indicates not done and >500 indicates that the selected compound was unable to kill 50% of the cells with the highest doses used. (Representative of n≥3)

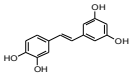
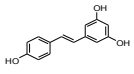
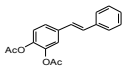
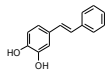
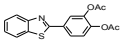
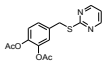
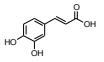
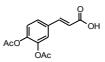
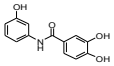
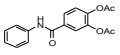
		EL	B Cell Lymphoma		Multiple Myeloma				TCL
	Compound	K562	Ramos	Raji	U266	OPM2	8226	MM1.S	Jurkat
	Piceatannol	155.3	3.9	0.7	8.8	31.4	25.6	56.5	9.3
	Resveratrol	nd	nd	nd	nd	nd	nd	nd	nd
	7	30.1	6.6	22.1	nd	nd	nd	nd	5.7
	29	91.1	32.0	45.1	19.8	74.2	nd	nd	24.6
	19	nd	nd	nd	nd	nd	nd	nd	nd
	31	156.6	63.7	79.5	nd	nd	nd	nd	23.2
	24	>500	469.3	>500	nd	nd	nd	nd	>500
	25	>500	500.0	>500	nd	nd	nd	nd	307.7
	3	430.7	79.0	34.5	nd	nd	nd	nd	23.4
	23	nd	nd	nd	nd	nd	nd	nd	nd

Table 5.2 – LC_{50} values for piceatannol-like compounds in human cancer cell lines of hematopoietic origin.

Human erythroleukemia cells (EL), B cell lymphoma cells, multiple myeloma cells, and T cell lymphoma cells (TCL) were treated for 48 hours with increasing concentrations of piceatannol-like compounds. Cell viability was determined by MTT, and normalized to untreated controls. LC_{50} values (μM) were calculated mathematically as described in Chapter 2. Note: nd indicates not done and >500 indicates that the selected compound was unable to kill 50% of the cells with the highest doses used. (Representative of $n \geq 3$)

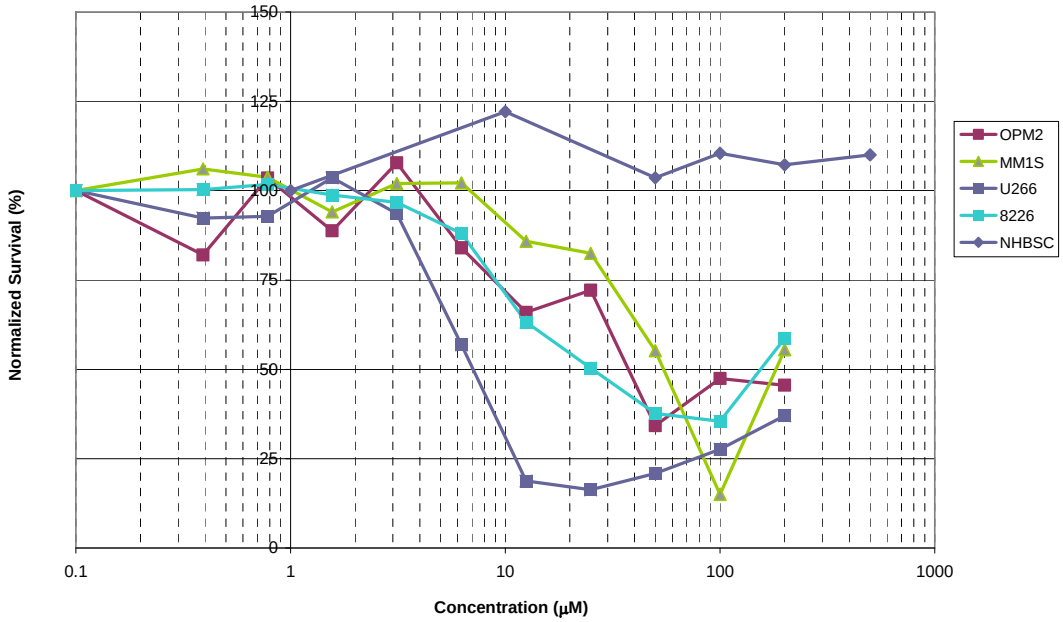


Figure 5.3 – Piceatannol exhibits a large therapeutic index for MM treatment.

Therapeutic index between primary normal human bone marrow stromal cells (NHBSC) and human multiple myeloma cell lines (OPM2, MM1.S, U266, and 8226). Cells were treated with increasing concentrations of piceatannol for 48 hours (NHBSC), or 72 hours (U266, OPM2, 8226, MM1.S). Cell viability was determined by MTT assay, and values were normalized to control DMSO treated cells. Each data point represents the mean of n=3. The minimum therapeutic index (TI) was estimated at 17 by using the average LC_{50} for the multiple myeloma cells of approximately 30μM and the maximum dose attempted for the NHBSC of 500μM (TI=500μM/30μM).

could only estimate the therapeutic index as the highest concentration of piceatannol used, 500μM, did not have any effect on the viability of the stromal cells. Therefore the minimal possible therapeutic index (TI) is approximately 17 (TI=500μM/30μM), given that piceatannol's average LC_{50} from the four human multiple myeloma cell lines tested was 30μM. The absence of toxicity I observed in normal primary human cells in culture is supported by several studies in which large doses of piceatannol were given to rodents without any adverse side-effects (Dang et al., 2004; Roupe et al., 2006).

5.2.3 Piceatannol induces the loss of the c-Cbl and Cbl-b proteins in Jurkat T cells

In addition to piceatannol's ability to kill MM cells, I was also interested in its ability to inhibit their CXCR4-mediated migration to the bone marrow niche. To determine if piceatannol could inhibit this process, I began by examining its effect on the Jurkat human T cell leukemia cell line, one of the most well characterized *in vitro* model systems of CXCL12/CXCR4-mediated migration. Western blots of lysates from Jurkat cells treated with increasing doses of piceatannol for increasing time periods indicated that piceatannol treatment lead to a dose and time dependent loss of both c-Cbl and Cbl-b (Figure 5.4). The concentrations of piceatannol used in this experiment where chosen to more accurately reflect the effects of concentrations realistically attainable *in vivo* and thus provide a better measure of whether piceatannol could be used as an effective therapeutic.

5.2.4 Piceatannol inhibits CXCL12-induced migration in Jurkat T cells

To determine if Cbl-loss correlated with impaired CXCR4-mediated signalling, and thus migration, I next examined the effect of piceatannol on CXCL12-induced Jurkat cell migration using a modified Boyden chamber. Cells were pre-treated with increasing concentrations of piceatannol for three hours prior to their introduction into a well, separated by a semi-permeable membrane from media containing increasing concentrations of CXCL12. After a one hour incubation with CXCL12, cells that had migrated through the membrane were counted and graphed (Figure 5.5). For all of the different concentrations of CXCL12 examined, 25μM piceatannol was sufficient to inhibit migration by approximately 50%. In Figure 5.4, Western blot data shows that at three hours post-treatment with 25μM piceatannol there is small reduction in c-Cbl levels

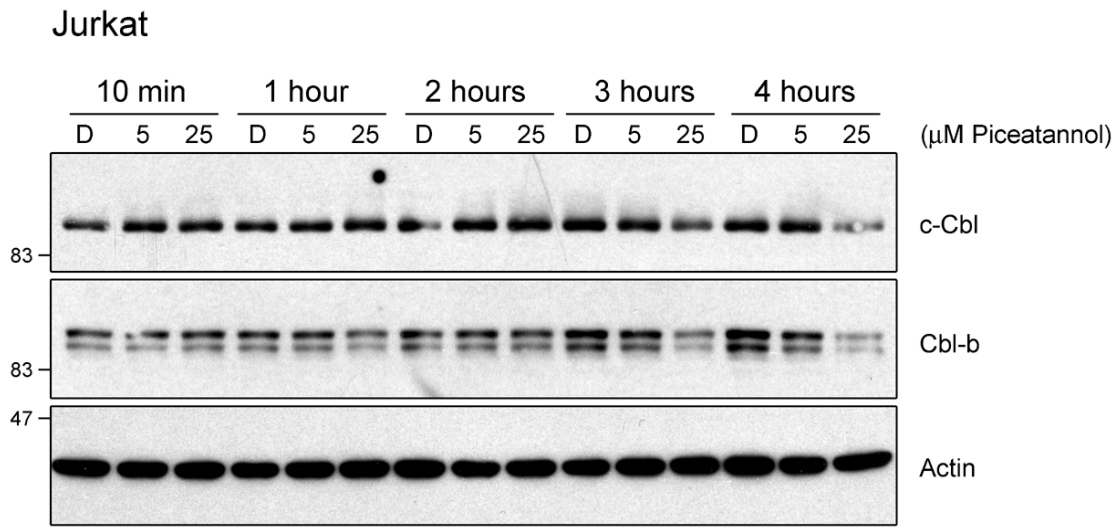


Figure 5.4 – Piceatannol induces Cbl and Cbl-b loss in Jurkat T cells.

Western blots of lysates from Jurkat cells that were treated for increasing time with DMSO (D), 5μM, or 25μM piceatannol. The top panel was probed for c-Cbl, the middle panel was probed for Cbl-b, and the lower panel was probed for actin as a loading control. (Representative of n=2)

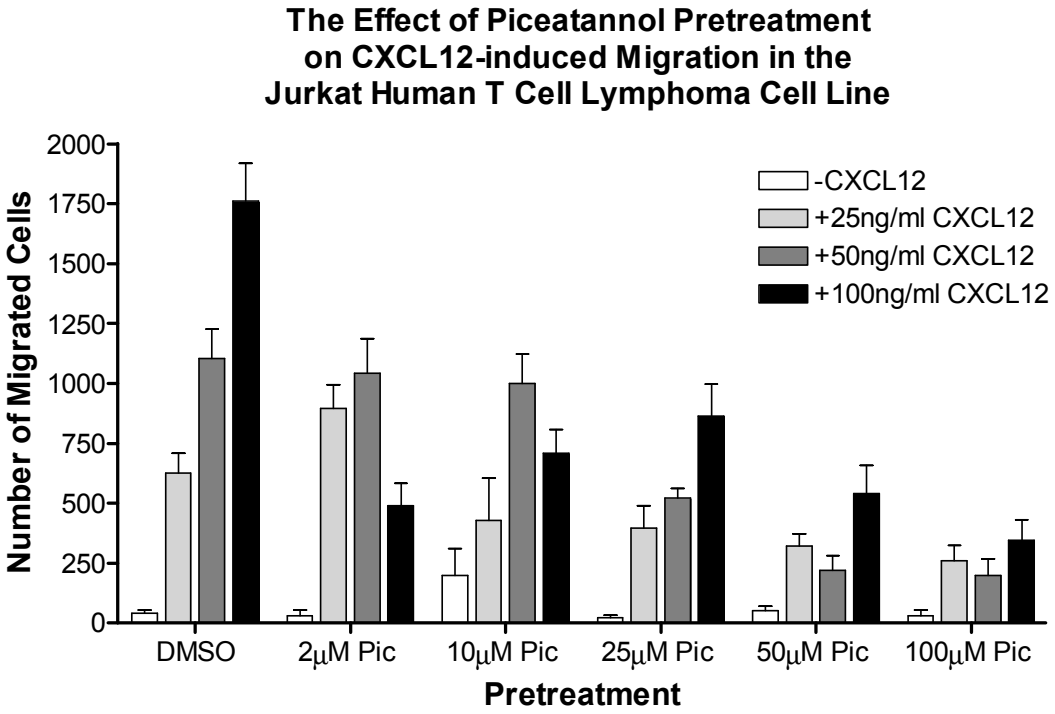


Figure 5.5 – Piceatannol inhibits CXCL12-induced migration in Jurkat T cells.
Graphical analysis of the effect of piceatannol pretreatment on CXCL12-induced migration in the Jurkat T Cell human lymphoma cell line. $1.25x10^4$ Jurkat cells were pretreated with DMSO, 2μM, 10μM, 25μM, 50μM, or 100μM piceatannol for 3 hours prior to a one hour migration period where the pretreated cells were placed on the other side of a 5μM pore semi-permeable membrane from media with 0ng/ml, 25ng/ml, 50ng/ml, or 100ng/ml of CXCL-12. Cells that migrated across the membrane were counted and graphed. Error bars represent standard error of the mean. (Representative of 6 replicates from 1 experiment)

and a moderate reduction in Cbl-b levels; by four hours, the reduction of c-Cbl and Cbl-b protein levels was more pronounced. The piceatannol-induced loss of Cbl protein therefore correlates strongly with the piceatannol-mediated inhibition of CXCL12-induced migration, which took place between the third and fourth hour post-treatment with piceatannol. This correlation is supported by Okabe *et al.*, these authors also saw an approximately 50% decrease in migration after decreasing either c-Cbl or Cbl-b levels through the use of siRNA (Okabe et al., 2006).

5.2.5 Piceatannol induces the loss of the c-Cbl and Cbl-b proteins in Multiple Myeloma cell lines

Having established a correlation between piceatannol-induced Cbl-loss and piceatannol-mediated inhibition of CXCL12-induced migration in Jurkat T cells, I proceeded to examine this phenomenon in a disease model. I initially screened through four different human multiple myeloma cell lines to see if piceatannol-induced Cbl-loss was a broadly applicable phenomenon in these cells. Treatment of MM cells with increasing concentrations of piceatannol for 3 hours lead to a dose responsive loss of Cbl-b, as determined by Western blotting (Figure 5.6). Of the four cell lines I examined, the 8226 and OPM2 cell lines had a more robust response to piceatannol than the MM1.S and U266 cell lines. I chose to focus on the OPM2 cell line due to its robust response to 25μM piceatannol, a concentration below piceatannol's 48 hour LC_{50} of 31.4μM (Table 5.2). As well, the OPM2 cell line was much more amenable to the modified Boyden chamber migration assay due to its low level of background migration (data not shown). Western blots of lysates from piceatannol treated OPM2 cells confirmed the ability of piceatannol to induce the loss of both c-Cbl and Cbl-b (Figure 5.7). I also examined the levels of the Cbl-associated protein Vav (Figure 5.7), which has been shown to mediate Rac activation and cytoskeletal changes associated with cell migration (Ma et al., 1998). Similar to what I observed with Cbl, piceatannol treatment lead to a dose dependent decrease in Vav levels, thus further supporting the hypothesis that piceatannol-induced protein loss could lead to the inhibition of CXCR4 signalling.

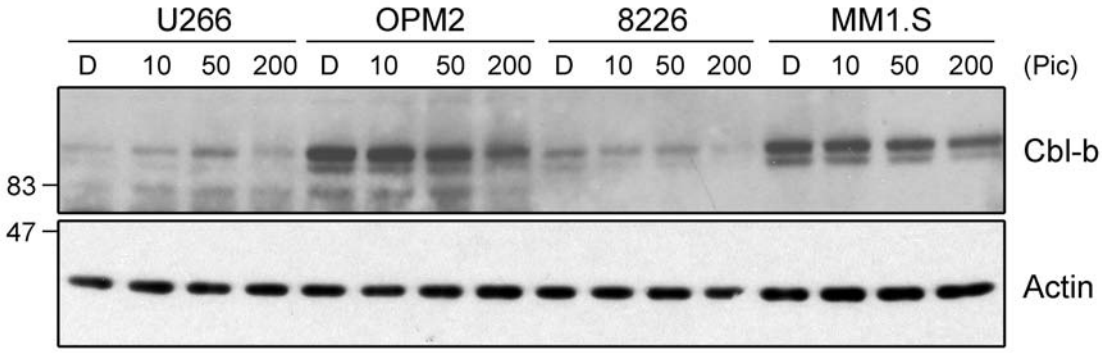


Figure 5.6 – Piceatannol induces Cbl-loss in MM cells.

Western blots of lysates from U266, OPM2, 8226, and MM1.S cells that were treated for 3 hours with DMSO (D), or increasing concentrations of piceatannol (10μM, 50μM, 200μM). The top panel was probed for the Cbl-b and the lower panel was probed for actin as a loading control. (Representative of n=2)

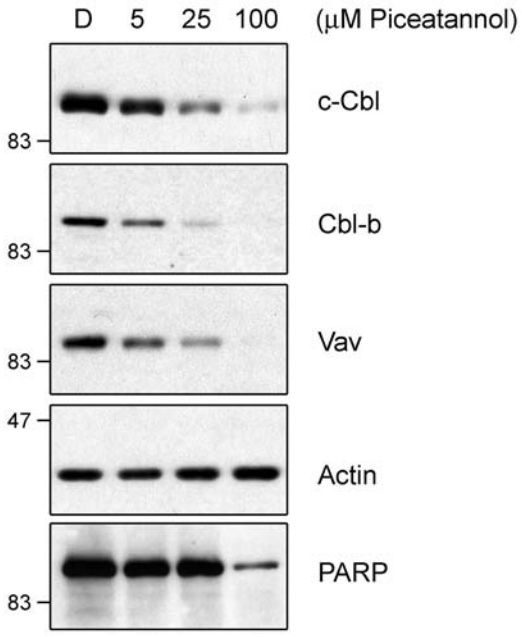


Figure 5.7 – Piceatannol induces the loss of Cbl, Cbl-b and Vav in the OPM2 human multiple myeloma cell line.

Western blots of lysates from OPM2 cells that were treated for 3 hours with DMSO (D), or increasing concentrations of piceatannol. The top panels were probed for the Cbl family members c-Cbl and Cbl-b, the middle panel was probed for the Cbl-associated Rac-GEF Vav, and the lower panels were probed for actin as a loading control and for PARP as an indicator of apoptosis. (Representative of n=2)

As piceatannol has been shown to induce apoptosis in a variety of cancer cell lines and apoptosis has been shown to induce the caspase-dependent loss of Cbl as well as other signalling molecules (Widmann et al., 1998), I performed experiments to ensure that the loss of Cbl and Cbl-associated proteins was not due to the induction of apoptosis. While I had already excluded the involvement of piceatannol-induced apoptosis and caspase activation in piceatannol-induced Cbl-loss in the 70Z/3 mouse B cell lymphoma cell line, it was necessary to ensure that piceatannol-induced apoptosis was not affecting the CXCL12-induced migration of OPM2 cells through the apoptosis-dependent inhibition of other signalling molecules involved in migration. Using PARP loss as a marker of caspase activation, I found no evidence of apoptosis at the concentrations of piceatannol and treatment time points used to examine CXCL-12 induced migration in MM cells (Figure 5.7).

5.2.6 Piceatannol inhibits CXCL12-induced migration in OPM2 multiple myeloma cells

I next examined the effect of piceatannol on CXCL12-induced OPM2 cell migration to determine if the piceatannol-induced Cbl-loss and Vav-loss observed in these cells correlated with impaired CXCR4-mediated migration. Cells were pre-treated with increasing concentrations of piceatannol for three hours prior to their introduction into a modified Boyden chamber with or without CXCL12. After a three hour incubation, migrated cells were counted and graphed (Figure 5.8). 25μM piceatannol significantly inhibited the migration of the OPM2 cells by over 60% (Figure 5.8), which correlated with an approximately 75% reduction in c-Cbl, Cbl-b, and Vav protein levels (Figure 5.7). 5μM piceatannol did not significantly inhibit migration of OPM2 cells (Figure 5.8), nor did it induce a dramatic reduction of c-Cbl, Cbl-b, or Vav protein levels (Figure 5.7). These data demonstrate that there is a strong correlation between the piceatannol-induced loss of signalling molecules known to be required for CXCR4-mediated chemokinesis and CXCL12-induced cell migration.

5.3 Discussion

In the previous chapter, I elucidated an oxidative mechanism responsible for many of piceatannol's anti-cancer properties. These properties include piceatannol's inhibition

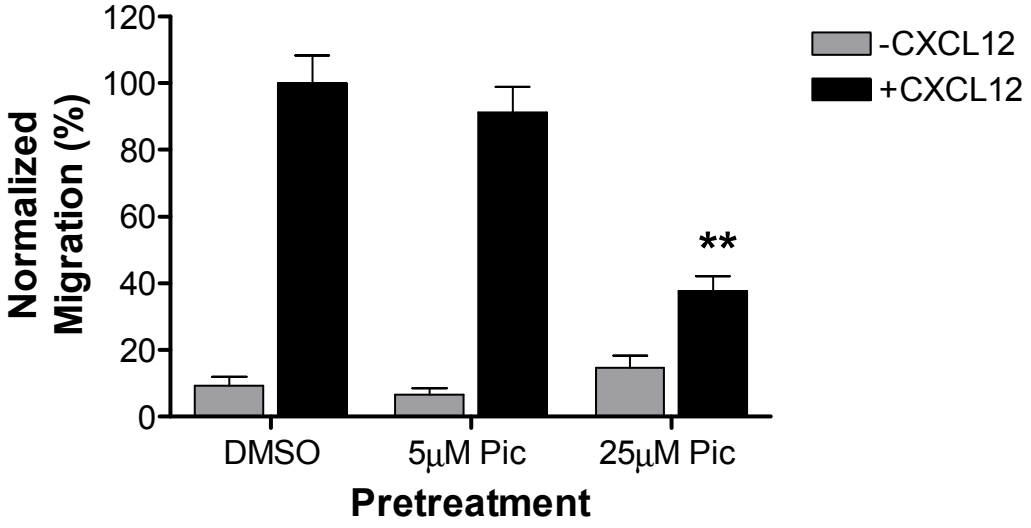


Figure 5.8 – Piceatannol inhibits CXCL12-induced migration in OPM2 cells.
Graphical analysis of the effect of piceatannol pretreatment on CXCL12-induced migration in the OPM2 human multiple myeloma cell line. OPM2 cells were pretreated with DMSO, 5μM, or 25μM piceatannol for 3 hours prior to a one hour migration period where the pretreated cells were placed on the other side of a 5μM pore semi-permeable membrane from media with or without 100ng/ml of CXCL-12. Cells that migrated across the membrane were counted and the total number of cells that migrated for each condition was normalized to the DMSO pretreatment in the presence of CXCL12 as 100%. ** indicates $p<0.001$ comparing DMSO and 25μM piceatannol pretreated, CXCL12 treated samples using a repeated measures ANOVA with Bonferroni's multiple comparison test. Error bars represent standard error of the mean. (Representative of 6 replicates each from 2 independent experiments)

of tyrosine kinases, its inhibition of matrix metallo-proteases, its initiation of DNA damage, and its induction of protein loss. Based on this data, I hypothesized that piceatannol would be a promising candidate for use as a cancer therapeutic. In this chapter I examined the feasibility of using piceatannol as a chemotherapeutic in a cell culture-based model system of multiple myeloma. To determine the feasibility of using piceatannol to treat multiple myeloma, I demonstrated that it has a large therapeutic index with respect to normal human cells (Figure 5.3) and I correlated its ability to induce Cbl-loss (Figures 5.4 and 5.7) with its ability to inhibit Cbl-dependent, CXCR4-mediated migration (Figures 5.5 and 5.8). In light of these positive results, further study to determine the possibility of synergy between piceatannol and current ROS generating chemotherapeutics and to determine the effectiveness of these treatments in an *in vivo* model of MM is warranted.

5.3.1 Piceatannol-induced cell death is selective for MM

Effective cancer therapeutics must demonstrate selectivity for cancer cells in their ability to induce cell death. I determined piceatannol's therapeutic index by dividing the LC_{50} values of primary normal human bone marrow stromal cells by the average LC_{50} of four human MM cell lines to demonstrate the feasibility of using piceatannol as a cancer therapy. The normal human stromal cells were unaffected by the highest concentrations of piceatannol used (Figure 5.3), generating a large therapeutic index that demonstrates a high degree of cancer selectivity. This high degree of selectivity for cancer cells demonstrated by piceatannol may be due to the fact that cancer cells generally have much higher ROS levels than normal cells (Toyokuni et al., 1995; Finkel, 2003; Loo, 2003). Therefore the oxidation dependence of piceatannol action, which I demonstrated in the previous chapter, would selectively target cancer cells with high levels of ROS.

The inability of piceatannol to affect normal cells also agrees with the absence of toxicity observed in animals treated with large daily doses of piceatannol (Dang et al., 2004; Roupe et al., 2006). While studies have not examined the effects of piceatannol in humans, the dietary intake of piceatannol and related polyphenols has generally been associated with beneficial health effects (Scalbert et al., 2005). Moderate consumption of red wine, a rich source of dietary piceatannol, has been correlated with the French

paradox (de Lange et al., 2007). The French paradox is the observation that the average French diet is high in fat, but their rate of coronary heart disease is lower than other northern European countries. It has been hypothesized that the polyphenols derived from red wine, widely consumed in France, are responsible for this cardio-protective effect (de Lange et al., 2007).

The only studies that demonstrate harmful side-effects from treatment with polyphenolic compounds are studies using very high doses of these compounds (Galati and O'Brien, 2004). Auto-antibodies against red blood cells and platelets were induced in humans taking cyanidol (also known as catechin) (Gandolfo et al., 1992), and hepatotoxicity has been observed in rodents receiving upwards 100mg/kg of EGCG, pyrogallol, and tannic acid. This is not surprising as such high doses would be expected to deliver mM concentrations of compound to the tissues of these animals, leading to numerous non-specific effects. 25-fold and 12-fold lower doses of EGCG and tannic acid, respectively, have been shown to have beneficial anticancer effects (Galati and O'Brien, 2004). The term lower dose, with respect to naturally occurring polyphenols, is a relative term since the LC_{50} values for these compounds in cell culture are generally in the μM range, whereas the LC_{50} values for many current chemotherapeutic agents in cell culture are in the nM range. The low LC_{50} values of current cancer therapeutics are general indications for their specificity for their defined targets and thus reduce the number and severity of side-effects resulting from off-target interactions. These low values also make it easier to deliver these compounds at therapeutic concentrations in animals and humans. Therefore, the LC_{50} value of 30μM for piceatannol in the human multiple myeloma cell lines is a relatively high LC_{50} for it to be an effective cancer therapeutic. I believe that this number can be further reduced through further screening of piceatannol-like molecules. The initial screen of compounds supports this idea: compared to piceatannol, compound 7 was able to reduce the LC_{50} of 3 of 4 breast cancer cell lines by more than 50% and of 11 of 15 cancer cell lines by at least 25% (Tables 5.1 and 5.2). The directed design of piceatannol-like molecules with the knowledge of the intended target may also allow us to take advantage of MM-selective processes to increase the potency of these compounds.

5.3.2 Piceatannol inhibits multiple myeloma migration

My interest in piceatannol as a therapy for multiple myeloma was more involved than simply taking advantage of its ability to induce the death of these malignant cells. I was also interested in the possibility of inhibiting MM migration to the bone marrow niche in response to CXCL12. By preventing myeloma cells from entering the bone marrow niche, it would be possible to reduce CAM-DR and prevent the disruption of bone homeostasis, which leads to the majority of the disease-associated morbidity (Hideshima and Anderson, 2002; Alsayed et al., 2006). I demonstrate that piceatannol can inhibit CXCL12-induced, CXCR4-mediated migration in both T cell leukemia and multiple myeloma cells (Figure 5.5 and 5.8). The inhibition of migration in these cells correlates with the piceatannol-induced reduction in c-Cbl, Cbl-b, and Vav proteins (Figures 5.4 and 5.7). While the link between inhibition of migration and Cbl-loss is only correlative, this observation is supported by a study demonstrating that siRNA knockdown of either c-Cbl or Cbl-b inhibited CXCL12-induced migration in Jurkat T cells (Okabe et al., 2006). It is further supported by a similar study that demonstrated that dominant negative Vav also inhibited CXCL12-induced migration (Vicente-Manzanares et al., 2005). Regardless of whether migration was inhibited by piceatannol-induced Cbl-loss or some other piceatannol-induced mechanism, including apoptosis, inhibition of CXCR4-mediated migration may on its own have therapeutic benefits to MM patients. Inhibition of migration to the bone marrow niche may improve sensitivity to other co-administered chemotherapeutic agents by reducing cell-adhesion mediated resistance and may also lead to improvements in quality of life by reducing the number and size of bone lesions (Hideshima and Anderson, 2002; Alsayed et al., 2006).

Apoptosis might also cause inhibition of migration by inducing the caspase-dependent loss of signalling and structural proteins involved in this process (Widmann et al., 1998; Geiser et al., 2004). To differentiate between the inhibition of migration due to piceatannol-induced Cbl-loss from piceatannol-induced cell death, I used concentrations of piceatannol in my migration experiments that were below the LC_{50} for multiple myeloma (Figure 5.8), and time points where there were no signs of apoptosis (Figure 5.7). Under these conditions, significant inhibition of migration was still observed

suggesting that this process is not necessarily dependent upon piceatannol-induced apoptosis. While this also indicates that piceatannol's inhibition of migration is mediated by piceatannol-induced Cbl-loss, the link I demonstrated in Chapter 4 between the mechanisms for piceatannol-induced Cbl-loss and piceatannol induced kinase inhibition hinders my ability to make these conclusions. Further studies using caspase inhibitors as well as comparative studies between piceatannol and other kinase inhibitors would help to eliminate these as possible mechanisms. It seems likely however, that a combination of all of piceatannol's properties are involved in its ability to kill MM cells and inhibit their migration.

5.3.3 Conclusions

I was interested in testing the feasibility of using piceatannol as a MM therapeutic. I initially demonstrated that MM cells are sensitive to piceatannol-induced cell death; however, I believed that its strength as an MM therapeutic would be derived from its ability to inhibit CXCR4-mediated migration of MM cells to the bone marrow. Newer MM treatments have demonstrated that by inhibiting the ability of MM cells to productively associate with stromal cells in the bone marrow microenvironment that they can increase the effectiveness of cytotoxic therapies. I hypothesized that piceatannol treatment of MM cells would inhibit their ability to migrate in response to CXCL12 due to the importance of c-Cbl and Cbl-b in CXCR4-mediated migration (Okabe et al., 2006). In addition to demonstrating that piceatannol treatment of MM cells induces the loss of Cbl proteins, I also show that it leads to the loss of the Cbl-associated Rac-GEF Vav (Figure 5.7), which is also required for CXCR4-mediated migration (Vicente-Manzanares et al., 2005). I correlate the loss of these critical effectors with the inhibition of CXCR4-mediated migration (Figure 5.8). I believe that piceatannol's ability to inhibit migration, coupled with its ability to kill multiple myeloma cells and its potential for synergy with current reactive oxygen species producing MM therapeutics, makes it an ideal candidate for further development as an MM adjuvant therapy.

Chapter Six: Summary

6.1 Cbl as a therapeutic target for the treatment of cancer

This thesis has helped to further clarify the mechanism of Cbl-mediated cellular transformation. Transforming mutants of Cbl act as oncogenic dominant negative proteins, leading to hyperactivated cellular signalling. I have shown that in order to behave as effective dominant negative proteins, and thereby promote cellular transformation, these Cbl mutants must have lost E3 ubiquitin-ligase activity as well as have gained the ability to constitutively associate with Cbl-substrates. I clarify that it is mutations within the Linker domain of Cbl that allow for both this loss- and gain-of-function. Further to the direct involvement of Cbl mutants in cellular transformation, I also show that constitutive phosphorylation of wtCbl, commonly found in cancer cells (Brizzi et al., 1998; Kamei et al., 2000), may be able to exacerbate cellular transformation. The constitutive phosphorylation of Cbl's Linker domain, mimics the unregulated loss- and gain-of-function seen with the transforming mutants of Cbl, and therefore could further exacerbate transformation. These data identify Cbl as a potential therapeutic target for cancer treatment.

To this end I have explored the Cbl-directed, therapeutic potential of piceatannol and piceatannol-like compounds. I have shown that cells treated with piceatannol and related compounds, exhibit the functional-loss of Cbl and Cbl-associated proteins. I determined that this loss was not accomplished through normal cellular Cbl-regulatory mechanisms; rather, it was directly mediated by oxidized piceatannol. The oxidation of piceatannol converts it into a highly reactive o-benzoquinone that is able to attack amino acid side chains as well as the peptide backbones of susceptible proteins, both *in vitro* and *in vivo*. The activation of piceatannol by oxidation, lends itself well to the use of piceatannol as a cancer therapeutic. Many cancer cells generate high levels of reactive oxygen species and many cancer treatments also further promote the production of reactive oxygen species (Loo, 2003). Therefore, piceatannol and piceatannol-like compounds may offer a significant therapeutic benefit in the treatment of cancer.

6.1.1 Wild-type Cbl and cellular transformation

In the process of elucidating the mechanism of Cbl-dependent cellular transformation I found that wtCbl may contribute to this process. I observed that the introduction of phosphomimetic mutations into Cbl's Linker domain allowed Cbl to promote cellular transformation (Figure 3.15), in spite of the fact that this type of mutation has been shown to enhance E3-ubiquitin ligase activity *in vitro* (Kassenbrock and Anderson, 2004). I propose that transformation mediated by phosphomimetic Cbl mutants is caused by the constitutive activation of the Cbl's TKB domain, resulting from the phosphomimetic mutations. Constitutive TKB domain activation blocks the ability of Cbl to cycle on and off of its substrates, preventing Cbl from releasing spent E2 ubiquitin-conjugating enzymes and recruiting fresh E2 ubiquitin conjugating enzymes (Figure 3.16). This inhibition of E2 enzyme turnover functionally blocks Cbl's E3 ubiquitin-ligase activity. Therefore, despite the fact that phosphomimetic Cbl mutants are able to mediate protein ubiquitination *in vitro*, their unregulated TKB domain activation functionally blocks their ability to mediate the ubiquitination and subsequent degradation of substrates *in vivo*. Thus Cbl proteins with phosphomimetic Linker domain mutations, due to their constitutively active TKB-domains, have functionally lost their E3 ubiquitin-ligase activity and thus conform to the dominant negative model of Cbl-mediated transformation. In a similar manner, wtCbl, when constitutively tyrosine phosphorylated in transformed cells, should constitutively associate with Cbl substrates and thereby functionally lose its E3 ubiquitin-ligase activity. The involvement of wtCbl in cellular transformation is supported by the observation that tyrosine phosphorylated Cbl is correlated with aggressive cancers (Brizzi et al., 1998; Kamei et al., 2000).

6.1.2 Piceatannol-induced Cbl loss

I have shown that piceatannol induces the selective *in vivo* loss of Cbl and specific Cbl-associated PTKs (Figures 4.31 and 4.32). Through a detailed examination of the experimental data I developed the following explanation for piceatannol-induced protein loss. Piceatannol is able to enter into cells and associate specifically with target proteins due to its small, rigid, planar structure. Intracellular oxidation converts piceatannol into an o-benzoquinone, which readily reacts with nucleophilic atoms from

Cbl's amino acid side chains (Ahlfors et al., 2003). This then leads to the cross-linking of Cbl, explaining the smearing of Cbl observed by Western blotting after piceatannol treatment (Waidyanatha et al., 1998; Boatman et al., 2000). Oxidized piceatannol can also react with Cbl's peptide backbone, inducing scission of the polypeptide backbone and leading to the loss of Cbl observed by Western blotting after piceatannol treatment (Dean et al., 1997) (Figure 4.19).

This explanation does not clearly account for the selectivity of piceatannol for specific proteins within the cell. It may be that the specific amino acid composition of these susceptible proteins provides a target within their primary structure. Peptides with defined amino acid sequences may be useful in this regard to define susceptible motifs. The observation that no specific region of Cbl is responsible for its sensitivity to piceatannol (Figure 4.2) suggests that many different amino acid sequences are susceptible to piceatannol attack. This observation also rules out the possibility that piceatannol-sensitivity is due to the direct association of piceatannol with specific elements of the higher order protein structure of target proteins.

The protein selectivity of piceatannol is more likely rooted in the cellular microenvironment of its target proteins. I have demonstrated the requirement for oxidation of piceatannol in order to induce the loss of Cbl and Cbl-associated proteins. It may be that the cellular microenvironment in which these proteins reside is oxidant-rich and thereby locally oxidizes and activates piceatannol. I propose the involvement of NADPH oxidases in the maintenance of these oxidant-rich microenvironments. NADPH oxidase complexes are known to be associated with signalling complexes as they promote sustained tyrosine phosphorylation by locally inhibiting protein tyrosine phosphatases. Therefore it is likely that Cbl, associated with signalling molecules in this microenvironment, would be highly susceptible to piceatannol-induced protein loss (Figure 4.36).

6.1.3 Piceatannol and piceatannol-like compounds as cancer therapeutics

Cancer therapeutics have evolved from relatively non-specific cytotoxic compounds to highly specific inhibitors of disease-initiating proteins. While these targeted therapeutics have been show to be effective in the treatment of cancer, and have

fewer side-effects than the early chemotherapeutics, they still have major flaws (Chabner and Roberts, 2005; Klein et al., 2005; Arbiser, 2007). Principally, targeting a specific disease-initiating protein allows for the development of cancer cell clones that are resistant to this therapy. This can occur either because these cancer cell clones have adapted to use multiple signalling pathways to promote their growth and survival, or because the targeted initiating-proteins can acquire resistance mutations, which allow them to evade the therapy (Chabner and Roberts, 2005; Arbiser, 2007). The use of piceatannol to target Cbl as cancer therapy addresses both of these issues. Since Cbl is involved in multiple, cancer associated, signalling pathways, this may give piceatannol a broad cancer specificity. Additionally, since I have shown that Cbl does not contain a specific piceatannol-sensitive region (Figure 4.2), this therapeutic strategy would not be sensitive to mutation-induced resistance.

I validated Cbl as therapeutic target for cancer and found that piceatannol and piceatannol-like compounds are able to target both Cbl and Cbl-associated oncogenic kinases (Figures 4.31 and 4.32). Using transformed cell lines I demonstrated that piceatannol is able to induce the loss of Cbl and Cbl-associated RTKs and subsequently inhibit their associated signalling pathways (Figures 4.33 and 4.34). These results provide support for revisiting piceatannol for use as an anti-cancer agent since many cancers are caused and/or aggravated by overactive kinase activity, and many of these kinases such as BCR-Abl, ErbB2, EGFR, and PDGFR are also Cbl-associated (Schmidt and Dikic, 2005). In addition to the inhibition of cellular signalling pathways in transformed cells, I validated the merit of using piceatannol-like compounds as a cancer therapy. To this end, I screened human cancer cell lines in order to identify cancer cells that were susceptible to piceatannol-induced cell death (Tables 5.1 and 5.2). I found that cancers of hematopoietic origin were particularly sensitive to piceatannol-induced cell death. I next postulated that multiple myeloma would be an ideal cancer model in which to evaluate the therapeutic potential of piceatannol because, not only was piceatannol able to induce apoptosis in myeloma cells (Table 5.2), but the bone-marrow homing and associated pathology in multiple myeloma proceeds through a Cbl-dependent migration process (Kuehl and Bergsagel, 2002; Alsayed et al., 2006; Okabe et al., 2006). Therefore

piceatannol treatment of multiple myeloma would induce the apoptosis of myeloma cells as well as inhibit any remaining myeloma cells from homing to the bone marrow and destroying the tissue. I demonstrated that the treatment of multiple myeloma cells with piceatannol at sub-apoptotic doses is able to inhibit the Cbl-dependent migration of these cells towards the bone marrow homing chemokine, CXCL12 (Figure 5.8). This inhibition of migration correlated with the loss of Cbl proteins in these cells (Figure 5.7). Additionally, since several chemotherapeutics, used for the treatment of multiple myeloma, are known to produce piceatannol-activating reactive oxygen species (Hideshima and Anderson, 2002), piceatannol may be an excellent therapeutic for the treatment of multiple myeloma in the context of combination therapies.

In conclusion, I have found that Cbl has potential as a therapeutic target for the treatment of cancer and that piceatannol-like compounds may be used to effectively treat cancers in which Cbl-associated signalling pathways are prominently involved in the progression of the disease.

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