The inhibitor of apoptosis (IAP) proteins contribute to acquired chemotherapy resistance in B-cell neoplasms

By

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Abstract

The addition of rituximab to the therapeutic regimens used to treat B-cell non-hodgkin lymphoma (B-NHL) has improved patient response rates, but it has also alter the biology of the disease in the relapse/refractory setting. The patients that relapse after receiving induction therapy containing rituximab respond less well to salvage chemotherapy regimens, and have a decreased overall survival rate. To study the molecular mechanisms controlling rituximab resistance in B-cell malignancies our group developed rituximab resistant cell lines (RRCL). These RRCLs are resistant to rituximab, as well as to chemotherapy agents. This resistance to chemotherapy agents closely mirrors what is observed clinically when lymphoma patients develop rituximab resistant disease.

The resistance to rituximab in RRCLs in mediated through down-regulation of CD20 (the target of rituximab), along with an upregulation of the complement inhibitory proteins CD55 and CD59. The molecular changes responsible for RRCL chemotherapy resistance involve alterations to the apoptotic response pathway, specifically loss of expression of the pro-apoptotic Bcl-2 proteins Bak and Bax. Without these two proteins RRCLs are highly resistant to any stimuli that activates the intrinsic apoptotic pathway, which is the cell death pathway triggered by many cytotoxic chemotherapy agents, including those used for the clinical management of lymphoma. These RRCLs also exhibit upregulated expression of survivin and livin, which are two members of the inhibitor of apoptosis protein (IAP) family. The IAPs act by directly inhibiting active caspases 3 and 7, which are the primary effectors of apoptosis. Our group previously determined that restoring expression of Bax in RRCLs can trigger spontaneous apoptosis, which indicated to us that restoring the balance of pro- and anti-apoptotic factors in RRCLs could improve anti-tumor activity of chemotherapy agents in these cells. Although forced expression of Bax was an effective strategy in RRCLs, it has little translational potential. The potential for IAPs to inhibit the effector caspases led us to hypothesize that targeting IAPs in
RRCLs would magnify the low level of natural apoptotic activity in these cells, and potential increase chemotherapy sensitivity.

To investigate if survivin or livin were regulating chemotherapy resistance in RRCLs we used a transient siRNA knockdown of each. Cells were then incubated with chemotherapy agents and the induction of apoptosis was measure through annexin-V/Sytox Blue staining. We observed no difference in the rates of apoptosis between Raji 4RH cells transfected with a scramble control, and those transfected with either the survivin or livin knockdown. We also observed no increase in apoptosis when we knocked down two additional IAPs, specifically cIAP1/2, either separately or together. When we used the same transient siRNA knockdown of XIAP we did observe a change in the rates of apoptosis. Knockdown of XIAP in the RRCL Raji 4RH increased the cytotoxic effect of both 50uM gemcitabine, as well as 50uM etoposide. Knockdown of XIAP in RL 4RH (another RRCL) was cytotoxic even without the addition of chemotherapy, and triggered spontaneous apoptosis measured by annexinV/Sytox Blue staining, as well as through western blot for PARP cleavage.

To determine if knockdown of XIAP could improve the anti-tumor effect of chemotherapy in vivo we generated a version of the Raji 4RH cell line with a stable knockdown of XIAP, and we implanted these cells into SCID mice at 10x10^6 cells per animal. Animals were treated with a combination of rituximab, ifosfamide, etoposide, and carboplatin (R-ICE). Animals implanted with Raji 4RH_XIAP KD responded to R-ICE chemotherapy better than animals implanted with the Raji 4RH_scramble control line; however, the results of this experiment were not repeatable. When we investigated the Raji 4RH_XIAP KD cell line we observed that over approximately 2 months in culture it had become chemotherapy resistant once again. We observed several protein changes in the newly resistant Raji 4RH_XIAP KD cell line, including overexpression of the IAP livin; however, XIAP remained knocked down, indicating that chemotherapy resistance in these new cells involves a novel mechanism.
The data indicating that XIAP knockdown can increase chemotherapy activity in RRCLs prompted us to investigate if small molecule IAP inhibitors could produce similar results. We selected two IAP inhibitors, LCL-161 and BMT-062789, each of which has reported inhibitory activity with XIAP. We exposed a panel of lymphoma cell lines, including RRCLs, to LCL-161 and BMT-062789 at escalating concentrations. LCL-161 single agent IC$_{50}$ concentrations were in the 20uM to 50uM range for most cell lines. Single agent IC$_{50}$ values for BMT-062789 were in the low micromolar range for most cell lines tested. BMT-062789 is a more selective XIAP inhibitor, and it appears to be active in lymphoma cell lines at lower concentration than LCL-161.

We also used western blot to investigate the how LCL-161 exposure impacted the expression levels of IAPs in RRCLs. The strongest effect was seen against cIAP1 in both the Raji 4RH and RL 4RH cell lines, which was totally depleted at 1uM concentration of LCL-161. The expression levels of survivin and livin were also decreased at higher concentrations of LCL-161. To determine if LCL-161 could enhance chemotherapy activity in RRCLs we cultured Raji 4RH cells with LCL-161 and various chemotherapy agents for 48 hours. We observed that LCL-161 synergistically enhanced the anti-tumor activity of etoposide, gemcitabine, and vincristine in the Raji 4RH cell line, with less synergistic responses seen in the Raji cell line. To determine if the combination of BMT-062789 and chemotherapy was more active than either alone we incubated RRCLs with BMT-062789 and etoposide. The rates of apoptosis (measured by annexin-V positivity) increased when BMT-062789 and etoposide were combined in both the Raji 4RH and RL 4RH cell lines.

To quantify the in vivo anti-tumor effect of LCL-161 we used a SCID mouse model implanted with a disseminated Raji 4RH tumor model. LCL-161 combined with chemotherapy produced a statistically significant increase long term survival compared to chemotherapy alone.
We also investigated the anti-tumor effect of LCL-161 and BMT-062789 in *ex vivo* lymphoma patient samples, and we observed that LCL-161 at a 10uM concentration enhanced the anti-tumor effect of carfilzomib in tissue samples from patients with a variety of lymphomas, including therapy relapse lymphomas. BMT-062789 at a 3uM concentration was active as a single agent in all *ex vivo* samples tested, and resulted in almost total tumor cell death, as measured by luminescent ATP assays. These results support our hypothesis that targeted XIAP inhibition with small molecule compounds is an effective anti-lymphoma strategy. In addition, selective XIAP inhibition appear to be a more effective anti-tumor approach in RRCL models.
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Chapter 1 – Introduction

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Lymphoma is a broad term used to describe a diverse set of cancers derived from the hematopoietic cell compartment in the body, although almost all lymphomas arise from either the B-cell, or T-cell lymphoid compartments. A major distinction in lymphoma classification, and biology, is the difference between Hodgkin’s lymphoma and non-Hodgkin’s lymphoma, or NHL.

Hodgkin’s lymphoma is a complex disease, which centers around neoplastic Reed-Sternberg cells (RS cells), which are a highly mutated B-cell. Hodgkin’s lymphoma is commonly divided into the classical Hodgkin’s subtype, and the lymphocytic-histiocytic subtype. Reed-Sternberg cells are the sole neoplastic cell type in each form of Hodgkin’s lymphoma, and in both cases they are completely reliant on a complex series of interactions with the tumor microenvironment, particularly follicular helper T cells.[1] Although Hodgkin’s lymphoma is a fascinating group of diseases, and it most certainly warrants ongoing investigation, this body of work is focused on the group of lymphomas commonly referred to as non-Hodgkin’s lymphoma.

Non-hodgkin’s lymphoma, or NHL, is a diverse group of cancers primarily derived from mature, or nearly mature B-cells. As the name would imply, NHL comprises all lymphomas which do not have the characteristics of Hodgkin’s lymphoma. The NIH estimates put new lymphoma cases in 2017 alone at over 70,000, and over 600,000 cases were reported worldwide.[2] Although NHL is far from the most prevalent cancer in the United States, it still represents a significant public health burden. Lymphomas can arise from either; however, B-cells are the cell of origin for most newly diagnosed lymphomas, around 90%, while T-cell lymphomas account for approximately 10% of newly diagnosed lymphomas in North America and Western Europe. The prevalence of the oncogenic T-cell leukemia virus in Southeast Asia increases the rates of T-cell lymphomas observed to around 20%.[3]
**B-cell NHL**

B-cell NHL (B-NHL) is by far the most common type of NHL, accounting for around 90% of newly diagnoses cases. B-NHL is commonly divided into many further subtypes based on cell of origin (surface markers) and genetic lesions.

The full list of B-NHL subtypes is extremely extensive; however, most of the cell line models discussed in this research are derived from the three main aggressive B-NHL subtypes. These are mantle cell lymphoma (MCL), Burkitt lymphoma (BL), and diffuse large B-cell lymphoma (DLBCL), which occurs as either the activated B-cell (ABC) or germinal center B-cell (GCB) subtype. Of these three types DLBCL is the most common and account for around 40% of all newly diagnosed lymphoma.

**Burkitt lymphoma (BL)**

Burkitt lymphoma is a fast growing form of lymphoma, which typically presents with a very homogeneous tumor micro environment. BL can be divided into three broad sub-classifications: endemic, sporadic, and immunodeficiency associated BL. The endemic form normally occurs in children in regions of Africa, and is commonly EBV positive. Although the clinical impact of endemic BL is low in North America and Western Europe, a number of the commonly used cell lines in lymphoma research are derived from patients with endemic BL, including the Raji cell line. The sporadic form of Burkitt lymphoma accounts most of the adult cases of BL diagnosed in North America and Western Europe (around 1%-2% of all adult lymphomas).[4] This form of BL can be either EBV positive or EBV negative. The immunodeficiency associated form of Burkitt lymphoma is usually observed in patients infected with HIV.
Most (approximately 80%) of Burkitt lymphomas carry the t(8:14) chromosomal translocation, which links expression of the c-Myc oncogene to the immunoglobulin heavy chain enhancer region. This leads to elevated, and continuous, c-Myc expression in transformed cells, which promotes unrestrained cell growth. Although the t(8:14) translocation is a commonly observed feature in BL, it is not a uniform genetic lesion of the disease. In the remaining 20% of BL cases that are negative for the t(8:14) translocation an alternative genetic lesion, either the t(2:8) or t(8:22), puts c-Myc expression under the control of one of the immunoglobulin light chain (kappa or lambda) regions.[4] One report described several cases of BL with no c-Myc translocation, but c-Myc was still overexpressed through an alternative mechanism involving micro RNA (miRNA) deregulation.[5] Although the molecular mechanisms differ, an overexpression of c-Myc is observed in essentially all cases of BL, and it appears to be the defining molecular characteristic of the disease.

Burkitt lymphoma is normally a very aggressive disease, but it also responds well to aggressive frontline therapy. Multi-agent chemotherapy regimens, such as the combination of cyclophosphamide, vincristine, and doxorubicin with methotrexate, ifosfamide, etoposide, and high dose cytarabine (CODOX-M/IVAC) have increased the rates of complete remission in BL at 1 year post therapy to above 80%.[4] The disease is still not uniformly cured in adults though, and additional therapies are still needed.

**Mantle cell lymphoma (MCL)**

Mantle cell lymphoma is another major classification of aggressive lymphoma, which makes up approximately 6% of all adult NHL.[6] The pattern of molecular alterations observed in MCL cases is much more diverse than BL, although the t(11:14) translocation leading to the overexpression of cyclin D1 is observed in most MCL cases, and is the genetic hallmark of the disease.[7] One retrospective study investigated the overexpression of cyclin D1 mRNA in...
cases of MCL through quantitative PCR (qPCR) and found overexpression of cyclin D1 mRNA in 92 of 101 total cases.[8] The high diversity of genetic alterations observed in MCL is most likely due to defects in the DNA damage response pathway in MCL. Mutations or deletions of the DNA damage response kinase ATM were observed in 35% of MCL cases in the same retrospective study mentioned above.[8] Deletions of TP53 have also been reported in MCL (11% of cases), although the authors noted that additional cases of MCL may contain TP53 inactivating mutations in the absence of complete gene deletion.[8] Additional genetic alterations in MCL include deletions of the INK4a/ARF tumor suppressor locus (21% of cases studied), which correlated with a high rate of tumor cell proliferation.

MCL is commonly treated with a combination of several chemotherapy agents given as a multi-agent therapy regimen. Although the standard of care for patients over 65 is the CHOP regimen combined with the anti-CD20 antibody rituximab, the more aggressive combination of hyperfractionated cyclophosphamide, rituximab, vincristine, doxorubicin, dexamethasone, and alternating cycles of cytarabine with methotrexate (Hyper CVAD/MA) has proven very effective in patients under 65.[9] Aggressive frontline induction therapy in MCL has increased completed response (CR) rates above 80% in some trails; however, long term OS rates in MCL are still low, with median survival rates between 5 and 7 years.[7] Novel therapies, such as BTK inhibitors and ubiquitin-proteasome system (UPS) disruptors represent promising new therapeutic avenues in MCL, but resistance to both these agents has begun to emerge, which will necessitate novel approaches to overcome therapeutic resistance.[10, 11]

**Diffuse large B-cell lymphoma (DLBCL)**

DLBCL is the most common form of lymphoma in adults. Cell lines derived from patients with DLBCL (such as RL, SU-DHL-4, and U2932) make up a sizable portion of the available *in vitro* models available for lymphoma research. DLBCL arises from mature B-cell that accumulate
genetic damage during germinal center maturation. Modern chemotherapy regimens have increased the long term survival rates for DLBCL to approximately 70%, but given the high prevalence of the disease the remaining 30% of patients that relapse represent a substantial public health burden. It is thought that the genetic damage which gives rise to DLBCL is an unfortunate side effect of the natural cytidine deaminase activity that occurs in the germinal center to promote class switching and affinity maturation of B-cells.[12] The exact pattern of genetic lesion observed in DLBCL cases appears to differ based on the stage of maturation the B-cell was in prior to the neoplastic transformation. Mutations in the CREB binding protein (CREBBP) gene are present in approximately 80% of DLBCL cases; CREBBP is believed to be responsible for defective germinal center maturation in DLBCL, and its inactivation leads to a perpetuation of premature germinal center genetic state in DLBCL. Another important B-cell maturation factor altered in GCB-DLBCL is BCL-6, which is overexpressed in around 75% of all DLBCL cases. Both of these alterations help maintain DLBCL cells in a genetically immature state and prevent terminal differentiation of these cells into memory B-cells or plasma cells. Although there are common mutations observed across most DLBCL cases advances in transcriptional profiling revealed some patterns in DLBCLs, which allowed the disease to be stratified into two subgroups; these are the germinal center B-cell DLBCL (GCB-DLBCL) subtype, and the activated B-cell DLBCL (ABC-DLBCL) subtype.[12] The classification of GCB vs. ABC in DLBCL is clinically relevant with long term event free survival rates differing significantly between the two groups (~75% for GCB-DLBCL and ~40% for ABC-DLBCL).[13]

One of the common mutations seen in the GCB-DLBCL subtype involves the t(14:18) translocation, which drives increased expression of the anti-apoptotic protein Bcl-2. This has been associated with an increased apoptotic threshold, and poor clinical response.[14] BCL-2 overexpression through the t(14:18) translocation is observed in around 30% of GCB-DLBCL cases. Another less prominent mutation seen in around 10% of GCB-DLBCL cases is the
t(8:14) translocation, which leads to an overexpression c-Myc.\[12\] In rare cases chromosomal translocations driving the overexpression of Bcl-2 and c-Myc can occur concurrently leading to what is known as double hit GCB-DLBCL. Additional mutations in the EZH2 methyltransferase enzyme have been reported in approximately 20% of GCB-DLBCL. EZH2 is broad acting epigenetic regulator responsible for regulating the expression of many genes, but potential targets of interest for EZH2 in GCB-DLBCL include genes involved in cell cycle regulation and maturation.

The ABC-DLBCL subtype is associated with many different mutations that generally lead to the same cellular process, specifically the activation of the NF-κB transcription factor pathway. Activation of the NF-κB pathway is a normal event in the activation and maturation of B-cells, but ABC-DLBCL is associated with chronic, unrestrained, NF-κB activation.\[15, 16\] This can be achieved through mutations in the B-cell receptor (BCR) signaling pathway, which drives NF-κB activation through Bruton’s tyrosine kinase (BTK). Additionally, mutations in the MYD88 protein, which normally transmits signals through the Toll-like receptor (TLR) pathway, can also promote activation of NF-κB. Approximately 30% of ABC-DLBCL cases also display inactivating mutations in the A20 enzyme, which is a negative regulator of NF-κB. Activation of the NF-κB signaling pathway has been shown to inhibit apoptosis in lymphoma cell lines.\[17\] Another common alteration seen in ABC-DLBCL is at the BCL2 locus, which encodes the anti-apoptotic Bcl-2 protein. Although chromosomal translocations driving the overexpression of Bcl-2 are not commonly observed in ABC-DLBCL, gain of function mutations are seen in approximately 30% of cases.

Generally the GCB subtype of DLBCL responded better to frontline therapy when compared to the ABC subtype. Data from the BIO-CORAL study demonstrated that GCB-DLBCL patients had a long term overall survival rate of approximately 70% after receiving frontline multi-agent chemotherapy, while ABC-DLBCL patients had a long term overall survival rate of
approximately 40%.[18] Although the prognosis for patients with ABC-DLBCL was historically somewhat poor, new agents to target the NF-κB pathway have either been approved, or are entering clinical trials. One of these agents, the ubiquitin proteasome inhibitor carfilzomib, is currently part of an ongoing clinical trial in relapsed/refractory DLBCL here at Roswell Park (NCT01959698).

**Rituximab therapy for B-cell NHL**

The therapeutic options to treat aggressive lymphomas, such as BL, MCL, and DLBCL have improved dramatically in the past three decades, and now cytotoxic chemotherapy regimens such as CHOP and Hyper CVAD/MA are commonly augmented with the anti-CD20 monoclonal antibody rituximab (R-CHOP and R-Hyper-CVAD/MA). This combined approach produced substantially improved complete response rates to frontline induction therapy. One trial in elderly patients demonstrated an increase in complete response rates from 37% in patients given CHOP alone to 52% in patients given R-CHOP.[19] Although rituximab improved survival for most DLBCL patients it also significantly altered the biology of B-cell NHL. Patients that relapse after receiving rituximab as part of their therapy, or are fully refractory to rituximab contained therapy regimens have a poorer overall response to commonly used lymphoma salvage therapies, such as the combination of etoposide, carboplatin, and ifosfamide (ICE or R-ICE when combined with rituximab).[20]

**Rituximab resistant cell lines**

To investigate how lymphoma disease biology was reacting to anti-CD20 therapy our group pioneered several rituximab resistant cell lines, which were derived from the normal B-NHL lines Raji (BL) and RL (GCB-DLBCL).[21] These rituximab resistant cell lines (RRCL) were created by exposing the sensitive parent lines to escalating levels of rituximab with human serum to provide complement.
Rituximab is thought to act through three primary anti-tumor mechanisms: antibody-dependent cell mediated cytotoxicity (ADCC), complement mediated cytotoxicity (CMC), and direct action through alteration of CD20 signaling. The RRCLs (Raji 4RH and RL 4RH) have accumulated several alterations, which make them less sensitive to rituximab compared to the sensitive parent cell lines Raji and RL. One of these mechanisms is a substantial downregulation of CD20 surface protein expression. In addition, both of these cell lines also expressed elevated levels of the complement inhibitor proteins CD55 and CD59, which blocked rituximab mediated complement activation.[22] Both of these observations were expected given the selection method used to create the cells, and represent the most direct cellular resistance mechanisms to anti-CD20 antibody exposure. Considering that these RRCLs had never been exposed to strong activators of the intrinsic apoptotic pathway, such as chemotherapy agents, we were surprised to find a number of anti-apoptotic mutations in the Bcl-2 family compartment in these RRCLs.[23] It is also worth noting that depleting CD20 from either the Raji or RL cell lines does not recreate the RRCL phenotype in either of these cell lines.[24] Repeated cycles of exposure to the cytotoxic stress of rituximab with human serum, followed by a recovery period, appears to be essential for the evolution of chemotherapy resistance in RRCLs. This most likely results from the gradual increase of cell stress over the 10 cycles of rituximab exposure the parent cells lines were subjected to, which would allow time for more complicated cellular alterations to arise, such as mutation in the apoptotic response pathways.

The cellular apoptotic response is a key regulator of tumor therapy resistance as it governs most of the potential cytotoxic pathways which can be exploited by chemotherapeutic agents. Resistance to apoptosis is a common molecular characteristic observed in many forms of lymphoma. This can occur either through direct overexpression of anti-apoptotic proteins like Bcl-2 through the t(14:18) chromosomal translocation, or as a result of increased activity of
transcription factors such as NFκB, which has been shown to upregulate the expression of the anti-apoptotic protein Bcl-X_L.[25]

The overall apoptotic response can be broken down into the intrinsic and extrinsic pathways, and although each pathway begins in a distinct area of the cell (the mitochondria and the cell membrane respectively) both culminate in the activation of the caspase cascade. The intrinsic pathway centers on the mitochondrial network, and is tightly regulated through the balance of pro and anti-apoptotic Bcl-2 family proteins, while the extrinsic pathway involves activation of cell surface death receptors such as Fas (CD95) and propagates the apoptotic signal through cleavage and activation of caspase 8. [26, 27] Although the extrinsic pathway is undeniably important in the anti-tumor action of antibody based therapies, such as rituximab, it is also able to influence the intrinsic apoptotic pathway through cleavage of the Bcl-2 family member BID. This interaction between the two apoptotic pathways allows a robust death receptor signal to influence mitochondrial stability, but it is not required for efficient initiation of apoptosis. [28] The importance of the extrinsic pathway to anti-tumor therapy is certainly well established and should not be discounted; however, the bulk of this work will focus on the intrinsic pathway due to the importance of the intrinsic pathway in responding to DNA damage and growth factor withdrawal, two of the key chemotherapeutic drug mechanisms of action.

The mitochondrial network is the core of the intrinsic apoptotic pathway. Loss of mitochondrial outer membrane integrity, through a process referred to as mitochondrial outer membrane permeabilization (MOMP), is the key event in programed cell death. Without a functional mitochondrial compartment a cell cannot function, and is functionally dead even if the rest of the apoptotic cascade is inhibited. [29] The fate of the mitochondrial network is, by necessity, carefully regulated. Most of this regulation is performed by the Bcl-2 family of proteins, short for B-cell lymphoma 2, which includes both pro and anti-apoptotic proteins with differing degrees of
homology to each other. [30] The Bcl-2 protein family is divided into three main subgroups based on function, as well as, structure.

The first group are the pro-apoptotic Bcl-2 proteins (Bax and Bak), which are responsible for forming pores in the mitochondrial outer membrane through an oligomerization process. Bak and Bax differ slightly in subcellular localization with Bak being mostly fixed in the mitochondrial outer membrane, while Bax moves more freely between the cytoplasm and the mitochondrial membrane. There is some evidence that the movement of Bax between the cytoplasm and mitochondrial compartments is important for other processes, however this work is very recent and may be a peculiarity of the model system used. [31]

The activation of the pro-apoptotic Bcl-2 proteins is tightly regulated by the remaining two Bcl-2 subfamilies, the anti-apoptotic Bcl-2 proteins (including Bcl-2, Bcl-XL, and Mcl-1) and the BH3 only proteins. The anti-apoptotic subfamily directly interact with either Bax and/or Bak to prevent oligomerization and restrain MOMP, while the BH3 only subfamily promotes apoptosis either by blocking the anti-apoptotic proteins, or through direct promotion of Bak and Bax oligomerization.[32] Overexpression of anti-apoptotic Bcl-2 proteins increases resistance to cytotoxic chemotherapy in cell lines[30], and correlates with poor survival outcomes in multiple cancer types, including lymphoma.[33-35]

The RRCLs Raji 4RH and RL 4RH exhibit significant alterations in Bcl-2 family expression compared to the sensitive parent cell lines. Each of the RRCLs is deficient in Bax expression due to a frame shift mutation, and has almost no expression of the other pro-apoptotic Bcl-2 protein Bak, which is partially the result of increased proteasomal activity.[23] Although these cells were selected for with rituximab and human serum only the loss of the pro-apoptotic Bcl-2 proteins renders these RRCLs highly resistant to all commonly used chemotherapy agents. This rituximab associated chemotherapy resistance mirrors what is observed clinically in
patients who have failed rituximab containing therapy regimens. The phenotypic similarities between clinical rituximab relapse/refractory lymphoma and RRCLs provide a unique opportunity to investigate how alterations in the apoptotic compartment downstream of the Bcl-2 family are affecting therapy response in these resistant lymphoma models with the eventual aim of identifying potential new therapeutic targets to exploit in rituximab relapse/refractory disease.

The loss of Bak and Bax effectively strengthens the mitochondria in RRCLs, and raises the threshold for induction of MOMP so high that clinically achievable concentrations of many chemotherapy agents have almost no effect on RRCL survival. In addition, both of the RRCLs exhibit increased expression of the anti-apoptotic Bcl-2 family members Bcl-XL and MCL-1, suggesting a model where numerous alterations in the Bcl-2 compartment are contributing to RRCL chemotherapy resistance. The extensive alterations in Bcl-2 family expression observed in RRCLs led our group to hypothesize that small molecule inhibitors of MCL-1 and Bcl-XL could increase the response to chemotherapy in RRCLs. To test this hypothesis our group used the small molecule obatoclax, which is classified as a BH3 mimetic based on its ability to mimic the action of the pro-apoptotic BH3 only Bcl-2 family proteins, such as Bad and Bim. Obatoclax is specifically reported to be a direct antagonist of both Bcl-XL as well as MCL-1. We determined that obatoclax was active in RRCLs, and had an anti-tumor effect in vitro in both RRCL models, as well as tumor biopsy samples taken from lymphoma patients at Roswell Park. Obatoclax also appeared to synergize with several chemotherapy agents commonly used for the clinical management of lymphoma. Although obatoclax clearly synergized with chemotherapy in RRCLs, the doses required to achieve full cell killing remained very high, probably in excess of what could be clinically achieved. This is mostly likely due to the very low expression of the essential pro-apoptotic Bcl-2 family proteins Bax and Bak. Without robust expression of either of these two proteins we felt that the response to BH3 mimetics in RRCLs would always be
somewhat limited, and this prompted us to investigate if other apoptotic regulatory networks may offer an alternative approach to restoring chemotherapy sensitivity in RRCLs.

**Figure 1.1 The importance of Bax and Bak in the apoptotic cascade**

a) Under normal conditions BH3 proteins (BAD, Bid, NOXA) antagonize the activity of the anti-apoptotic Bcl-2 proteins (Bcl-2, Mcl1). This releases Bak and Bax, and promotes the formations of pores in the mitochondrial outer membrane through Bak/Bax oligomerization. b) If Bax and Bak are not expressed at sufficient levels even full antagonization of the anti-apoptotic Bcl-2 proteins will not trigger apoptosis. Loss of Bax and Bak makes a cell highly resistant cytotoxins, which alter the levels of natural BH3 proteins, as well as small molecule BH3 mimetics.

begins at the mitochondria, and is mediated through Bcl-2 family interactions, but the functional outcome is activation of the apoptotic effector caspases, specifically caspases 3 and 7, which are activated by caspases 8 and 9, the two initiator caspases. Caspase 8 is the initiator of cell extrinsic apoptosis activated through death receptor signaling, while caspase 9 governs the so-called intrinsic apoptotic pathway, which centers on the mitochondria. Other caspases, specifically caspases 2 and 6 have also been described as having an effector caspase role, but this is probably more of a model specific observation, and less of a general function. In either cases, caspases 3 and 7 are primarily responsible for proteolytically cleaving downstream substrates such as poly-ADP-ribosyltransferase (PARP), activating DNA cleavage, and fragmenting the nuclear membrane; all of these processes are important for the ordered dismantling of the cell. The activity of these effector caspases is, by necessity, tightly controlled
by a second family of apoptotic regulatory proteins. The inhibitor of apoptosis protein family (IAP) control apoptosis through direct interaction with the activated forms of caspases 3, 7, and 9, and our initial studies indicate that several of these IAPs, specifically survivin and livin, are upregulated in RRCL models compared to parent cell lines. The results led us to hypothesize that the IAP protein family was regulating chemotherapy resistance in RRCLs, and that targeted inhibition of IAPs could restore RRCL chemotherapy sensitivity.

**The IAP protein family**

The IAP protein family consists of 8 proteins, all of which have at least one baculovirus IAP repeat (BIR) domain. This BIR domain is responsible for the alternate name for the protein family, the baculovirus IAP repeat domain contain proteins (BIRC). Beyond this single thread of homogeneity, the IAP family is highly variable in terms of both size and function. Five of the IAPs also have a RING (relay interesting new gene) E3 ubiquitin ligase domain, which is important for a number of functions including regulating NFκB activation through the tumor necrosis factor receptor (TRFR1), and controlling TGFβ signaling through JNK1 kinase inhibition.[37, 38] The IAP BRUCE also has a non-RING ubiquitin ligase domain, although its function is less well studied. The IAPs with the most well defined anti-apoptotic functionality are cIAP1 (BIRC2), cIAP2 (BIRC3), XIAP (BIRC4), survivin (BIRC5), and livin (BIRC7). ILP-2 (BIRC8) and BRUCE (BIRC6) have also been reported to have anti-apoptotic activity, but are less well studied.[38] The final IAP family member is the neuronal apoptosis inhibitory protein (NAIP, also known as BIRC1). The importance of IAPs in hematological malignancies has been characterized through both retrospective patient tissue studies, as well as direct experiments involving both in vitro and in vivo models.
The structure of the IAP family proteins

Figure 1.2 The structure of the IAP family proteins

BIR1, BIR2, and BIR3 domains indicate the baculovirus IAP repeat domains. All the IAP family members contain at least one BIR domain; however, NAIP, cIAP1/2, and XIAP contain 3. NAIP also includes a nucleotide-binding oligomerization domain (NOD), which functions in regulating inflammasome formation. The caspase recruitment domain (CARD) is present on only cIAP1/2, although its full function is still incompletely understood. The really interesting new gene (RING) domain is present on cIAP1/2, XIAP, livin, and ILP-2. RING functions as an E3 ubiquitin ligase. The IAP BRUCE contains a similar ubiquitin conjugation domain (UBC). The coiled-coiled domain is unique to survivin, and is reported to be important in the function of survivin at the centromere.

cIAP1/2

The closely related cIAP1 and cIAP2 proteins are probably the most widely studied members of the family. They are both around 600 amino acids long, although both have additional alternatively spliced isoforms. The cIAP1/2 proteins contain all three of the BIR domains expressed in the IAP family, along with the RING E3 ubiquitin ligase domain. Additionally, the cIAP1/2 proteins contain a CARD caspase recruitment domain, which is not expressed on any of the other IAP family members.[39] The cIAP1/2 proteins were originally thought to directly bind and inhibit active caspases like the X-linked inhibitor of apoptosis (XIAP); however, this turned out to be an artifact of early in vitro experiments and consistent with the in vivo activity of cIAP1/2.[40] Even though cIAP1/2 do not stably bind caspases they both have well documented anti-apoptotic activity, which is thought to involve ubiquitin conjugation to caspases through the RING E3 ligase domain.[39] Another important function of the cIAP1/2 proteins is in regulation of TNF (tumor necrosis factor) receptor family signaling, which also happens to depend on the RING domain.
The cIAP1/2 proteins form an important portion of the intracellular signaling domain of the TNFα receptor. They auto-ubiquitinylate themselves upon binding the intracellular tail of the TNFα receptor. The ubiquitin chains of cIAP1/2 then act as binding domains for downstream signal transduction proteins important for both canonical and non-canonical TNF receptor family signaling, although the specific importance of cIAP1/2 is different in the two contexts.

Figure 1.3 Small molecule compounds available to target IAPs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Creator</th>
<th>Structure</th>
<th>IAP Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCL-161</td>
<td>Novartis</td>
<td>Monovalent SMAC mimetic</td>
<td>cIAP1, cIAP2, XIAP, livin</td>
</tr>
<tr>
<td>BV6</td>
<td>Genentech</td>
<td>Bivalent, homodimeric SMAC mimetic</td>
<td>cIAP1, cIAP2, XIAP, livin</td>
</tr>
<tr>
<td>TL32711</td>
<td>Tetralogic</td>
<td>Bivalent, homodimeric SMAC mimetic</td>
<td>cIAP1, cIAP2, XIAP, livin</td>
</tr>
<tr>
<td>CS3</td>
<td>Genentech</td>
<td>Monovalent SMAC mimetic</td>
<td>cIAP1, cIAP2</td>
</tr>
<tr>
<td>compound 36</td>
<td>Hoffmann-La Roche</td>
<td>Monovalent benzodiazepinone</td>
<td>XIAP BIR2 domin</td>
</tr>
<tr>
<td>BMTY-062789</td>
<td>Bristol-Myers Squibb</td>
<td>Bivalent, heterodimeric SMAC mimetic</td>
<td>XIAP selective</td>
</tr>
</tbody>
</table>

Ubiquitinylated cIAP1/2 acts as a positive regulator of canonical TNF receptor signaling, and promotes the phosphorylation and degradation of IκB to release the active p50:RelA complex. When cIAP1/2 are removed from this pathway TNFα signaling shifts to a pro-apoptotic event. In contrast to canonical signaling, cIAP1/2 directly inhibit non-canonical NFκB signaling by ubiquitinylating the NIK kinase, which is critical to the processing of p100 into p52, which then complexes with RelB.\[41\] This aspect of cIAP1/2 biology is especially important with regard to IAPs as a therapeutic target. The first compounds designed to target IAPs specifically targeted LCL-161, BV6, and TL32711 were some of the first SMAC mimetics developed. They biochemically interact with cIAP1/2, XIAP, and livin, but are most effective at depleting cIAP1 in cells. The selective IAP inhibitors were synthesized more recently, and represent an evolution of the medicinal chemistry of the field. CS3 is the best described cIAP1/2 selective agent created to date. Compound 36 is the only small molecule that had specificity for the XIAP BIR2 domain region, which is responsible for binding active caspases 3 and 7. BMT-062789 is a bivalent IAP inhibitor, which is composed of two similar, but distinct molecules joined together. It has binding affinity for both the BIR3 and BIR2 regions of XIAP, which allows it to antagonize the interaction of XIAP with active caspases 3, 7, and 9. BMT-062789 is also somewhat selective for XIAP over cIAP1/2.
ciAP1 with the goal of shifting TNF receptor signaling from an NFκB activating event to a cytotoxic event.[41]

The clinical significance of the ciAP1/2 expression in the context of hematological malignancies has been reasonably well studied, although the importance of each appears to be very context dependent. The clearest role for ciAP1/2 in driving tumor growth is seen in MALT (mucosa-associated lymphoid tissue) lymphoma subtype; ciAP2 is fused to the MALT1 proteins through the 11:18 gene translocation to produce a fusion protein, which drives chronic NFκB signaling. This chronic NFκB acts as a primary driver of MALT lymphoma tumor growth.[42] In addition, increased ciAP2 expression was shown to correlate with decreased overall survival in newly diagnosed AML patients.[43] The opposing roles for the ciAP1/2 proteins in both the canonical and non-canonical NFκB signaling pathways mean that the role of ciAP1/2 in tumor growth and survival is most likely going to be very disease context dependent and this can be seen clearly in multiple myeloma where deletions were observed in ciAP1 and ciAP2, which were shown to activate the non-canonical NFκB pathway. In this setting the non-canonical NFκB pathway appears to be more important for tumor cell survival;[44] however, another study of multiple myeloma patient samples demonstrated that ciAP1/2 were upregulated in patients whom failed chemotherapy treatment.[45] This suggests that loss of ciAP1/2 can promote tumor growth through activation of the non-canonical NFκB pathway, but may also make a tumor more sensitive to treatment.

**XIAP**

Other than the ciAP1/2 proteins, XIAP (x-linked inhibitor of apoptosis) is probably the most well studied of the IAP family. It is similar in both size and composition to the ciAP1/2 proteins, although XIAP has no CARD domain. XIAP is also unique among the IAP family members for its ability to bind and sequester the active forms of caspases 3, 7, and 9 under in vivo conditions.
conditions. Another key difference between the cIAP1/2 proteins and XIAP is the functionality of the RING domain, which is expressed on all three. Although the E3 ligase activity of the RING domain is important for all three, the specific role for the RING domain of XIAP is somewhat different for cIAP1/2, where E3 ligase activity is central for protein function. XIAP E3 ligase activity has been shown to contribute to the anti-apoptotic functioning of XIAP through degradation of the endogenous XIAP inhibitor SMAC[46], and it may have some NFκB stimulatory activity in solid tumors[47], but it is not a central component in the TNF receptor pathway like the cIAP1/2 proteins.

The clinical significance of elevated XIAP expression has been well established at this point, and it correlates with poorer overall survival in most adult hematological malignancies. Specifically, higher XIAP expression correlated with decreased survival in AML (acute myeloid leukemia), CLL (chronic lymphocytic leukemia, and DLBCL (diffuses large B-cell lymphoma).[42] In contrast to cIAP1/2 where ambiguity exists as to how elevated protein expression correlates with patient survival, the clinical significance of elevated XIAP expression in hematological malignancies is more uniformly negative.

**Survivin**

Survivin, or BIRC5, is the smallest of the IAP family with a total size of 140 amino acids, although several shorter splice isoforms have been reported. The protein’s total size of approximately 20kd, and its somewhat bizarre structure compared to the other IAP family members make survivin arguably the most unique IAP.[39] Unlike XIAP and the cIAP1/2 proteins, survivin has only one BIR domain, and does not functionally inhibit caspases on its own.[5, 48] This does not mean that survivin has no role in regulating apoptosis. Survivin is thought to inhibit the endogenous IAP antagonist SMAC (explained in detail below).
inhibiting SMAC, survivin is thought to boost the caspase binding anti-apoptotic activity of XIAP.[48]

Although it has the potential to regulate apoptosis, the principle function of survivin appears to be regulating cell mitosis. Survivin is essential for assembly of the chromosomal passenger complex, which is a necessary step in cytokinesis. The IAP BRUCE is also reported to be involved in the regulation of cytokinesis through formation of a complex with survivin.[49]

Survivin is normally not expressed at high levels in healthy adult tissues. This is in stark contrast to neoplastic cells, which generally expression high levels of survivin. Hodgkin’s lymphoma, non-Hodgkin’s lymphoma, leukemias, and many solid tumor types are reported to express high levels of survivin.[50] In addition, increased expression of survivin in many solid tumor types has been linked to poorer overall survival in retrospective trials.[50]

Livin

ML-IAP (melanoma-IPA), BIRC7, or livin, is one of the smaller IAPs. It normally is expressed in two isoforms, each in the 35kDa range. Like survivin, livin has only one BIR domain, and does not directly inhibit caspases, although it has been shown to be anti-apoptotic. Livin also expresses the RING E3 ligase domain, and has been shown to promote the degradation of SMAC through ubiquitin ligation.[51] This most likely accounts for the observed anti-apoptotic activity of livin. Genetic knock-down studies have demonstrated that silencing livin can improve chemotherapy response in cell lines[52]; however, the impact of livin expression on apoptotic resistance is probably highly model specific. This view point is supported by the very mixed clinical reports concerning the impact of livin expression in tumors.[53]
NAIP

The neuronal apoptosis inhibitory protein, or NAIP, is the second largest (160kD) of the IAP family. Like cIAP1/2 and XIAP, NAIP has all three of the BIR domains, and has been reported to have anti-apoptotic activity.[54] Although NAIP may have anti-apoptotic functionality the normal function of the protein appears to be in assembly of the NOD like receptor inflammasome signaling pathway, which supports innate immune signaling.[55] Like survivin, NAIP has also been shown to have a role in cytokinesis; however, these results are still new, and they may turn out to be model specific.[56]

ILP-2

Very little is known about ILP-2 compared to the other IAP family members, although it appears to be the newest member of the IAP family (evolutionarily speaking). ILP-2 is only present in great apes, and even then it is expressed primarily in the testis where it is believed to play an important role in sperm development.[57] No direct link between ILP-2 and human cancer has been definitively established at this point, but neoplastic cells can have a highly deregulated proteome with expression of proteins not normally present in the tissue type of origin. ILP-2 has anti-apoptotic activity, at least in vitro, and it is not out of the realm of possibility that ILP-2 could be an anti-apoptotic factor in human tumors, but again, this is only speculation.

SMAC and HtrA2/Omi

The cellular apoptotic system functions through a series of protein:protein interactions, which balance the effect of pro-apoptotic proteins against anti-apoptotic proteins to ensure the cell is viable, but also ready to respond to cell death stimuli. Just as the effect of anti-apoptotic Bcl-2
family proteins is balanced by the BH3 only group, the caspase inhibitor activity of the IAP family is controlled by the endogenous IAP inhibitors. SMAC (second mitochondria-derived activator of caspases) is by far the most well studied member of this group. SAMC is a relatively small (25kDa) protein that takes on its full functional conformation after being processed in the mitochondria where its mitochondrial localization sequence is removed through proteolytic cleavage. After processing, SMAC is retained inside the mitochondrial until it is released as part of the same Bax/Bak mediated process of MOMP (mitochondrial outer membrane permeabilization) that also controls cytochrome C release. Once released into the general cytoplasmic space SMAC promotes apoptosis by directly competing with caspases at the IAP caspase binding domains.[58] This happens through the SMAC AVPI peptide sequence, which is exposed after SMAC procession in the mitochondria. The AVPI sequence is reported to target the BIR3 IAP domain, which is important for binding caspase 9.[39] SMAC can also disrupt the caspase 3/7 bind activity of XIAP, but the exact biochemical mechanism is still poorly defined. The binding of SMAC to cIAP1/2 can also promote IAP auto-ubiquitinylation, which leads to proteasomal degradation.[41] In theory other IAP proteins with a RING domain could also be affected by SMAC in this way, but cIAP1/2 appear to be much more sensitive to this mechanism of depletion. Despite its clear role in regulating the apoptotic response there is comparatively little reported on the impact of altered SMAC expression in human disease, although one retrospective trial in non-small cell lung cancer (NSCLC) did show a correlation between higher SMAC expression and improved survival.[59]

HtrA2 (high temperature requirement A2) is a highly conserved member of a highly conserved family of proteins in both bacteria and eukaryotic cells. The primary function of HtrA2 appears to be in mitochondrial maintenance. This model is supported by observations that mice which express a protease defective version of HtrA2 experience marked neurodegeneration due to mitochondrial disfunction.[60] Along with its role mitochondrial maintenance HtrA2 also plays a
role in regulating apoptosis through direct interaction with IAPs. This is facilitated through the HtrA2 IAP binding region, which is similar to the IAP binding domain on SMAC. Unlike SMAC, HtrA2 has an additional serine protease domain, which allows it to directly target IAPs, and in theory would allow HtrA2 to degrade even the IAPs which do not have the RING E3 ligase domain required for auto-ubiquitination.

**Regulation of apoptosis by IAPs**

Although the structure and functional domains of SMAC and HtrA2 are somewhat different, functionally they are very similar. Both are released from the mitochondria following the induction of MOMP, and both exert their effects by targeting IAPs to increase the free caspase levels in the cytoplasm. Although the biochemical mechanisms of action differ between SMAC and HtrA2, functionally they are mostly the same. This is a theme that repeats throughout the broader IAP compartment. Although each individual protein has distinct properties there is a substantial degree of functional redundancy. This has been demonstrated quite strikingly through the use of IAP genetic knockout mouse models where the only lethal single knockout IAP mutations are loss of survivin or BRUCE. Both of these result in embryonic cells that fail to divide, not an uncontrolled cell death event. Even loss of XIAP alone is not sufficient to induce uncontrolled cell death, but loss of XIAP in addition to cIAP1 is embryonic lethal (as is cIAP1/2 double knockout).[61] This functional redundancy is observed in the Bcl-2 protein family as well, and was probably a useful evolutionary adaptation that allowed many pro-survival and pro-death factors to be balanced simultaneously through a single response pathway. Although most diagrams of the apoptotic regulatory pathway depict discrete interactions
between two proteins resulting in a binary outcome, a more correct model is one of opposing factors weighed against each other where the intensity of the signal determines the outcome. This model helps to explain how multiple IAPs have well demonstrated in vitro anti-apoptotic activity, but are unable to stably bind caspases. Through inhibition of SAMC or HtrA2 any IAP can boost the caspase binding activity of XIAP by shifting the overall balance of pro- and anti-apoptotic signals.
Small molecule IAP inhibitors

Although some attempts have been made to target IAPs with anti-sense RNA, the most widely investigated method of IAP inhibition involves SMAC mimetics, which are small molecule inhibitors designed to mimic the AVPI peptide sequence in SMAC. Although these compounds are much smaller than the SMAC protein in many regards they can mimic the activity of SMAC at the BIR3 IAP domain, which is why they are also referred to as SMAC mimetics. The BIR3 domain is the binding site for cleaved caspase 9 on XIAP, but the domain itself is present on additional IAPs, most importantly cIAP1/2. Upon binding to the BIR3 domain, SMAC mimetics can promote several different events concurrently. The most direct of these is increasing the amount of free caspase 9 in cells through competitive inhibition at the BIR3 domain on XIAP. An alternative mechanism of SMAC mimetic anti-tumor activity is boosting the levels of free SMAC through the same competitive inhibition of the BIR3 domain. The third main mechanism of action for SMAC mimetics is the targeted depletion of cIAP1 through induced auto-ubiquitinylation. Although cIAP2 can be targeted through the same mechanism, it appears to be more resistant to this process in most model systems. Loss of cIAP1/2 serves to increase free SMAC in a cell, but it is also the principle event in switching TNF receptor signaling to a cytotoxic outcome. In some lymphoma cell line models (WSU-DLCL2, Karpas 422) this mechanism is highly active, and SMAC mimetics are very effective inducers of cell death on their own with IC$_{50}$ values in the low to sub-micromolar concentrations. Unfortunately, these cells are more the exception, rather than the rule. Most lymphoma cells lines are highly resistant to SMAC mimetics when used alone, and significant anti-tumor effect is only observed when SMAC mimetics are combined with additional therapies.
Rationale and Specific Aims

Rituximab has proven to be an extremely valuable tool for the management of both indolent and aggressive lymphomas, but many patients will eventually relapse after being treated with a rituximab containing treatment regimen. These patients show a marked decrease in response to salvage therapy compared to rituximab naive patients. To better understand why rituximab resistance correlates with chemotherapy resistance our group has developed rituximab resistant lymphoma cell lines (RRCL). RRCLs show deregulation of the normal apoptotic BCL-2 and Inhibitor of Apoptosis (IAP) protein families when compared to their rituximab sensitive parent cells. RRCL also show substantially increased resistance to many chemotherapy drugs. The inhibitor of apoptosis (IAP) proteins are important regulators of the normal cellular apoptotic response pathways, and overexpression of IAP proteins has been shown to make cell resistant to cell death stimuli. IAPs act downstream of the BCL-2 protein family, and can directly bind active caspases. This direct inhibition of active caspases makes the IAP proteins the last checkpoint before full caspase activation. Initial characterization of the expression levels of IAP proteins in RRCLs showed that two IAPs, survivin and livin, were strongly up regulated in the RRCLs compared to sensitive parent cells. The two specific aims of this thesis were to determine if IAP family member proteins are contributing to rituximab/chemotherapy resistance in RRCLs, and to investigate the anti-tumor activity of small molecule IAP inhibitors (SMAC mimetics) in RRCL models.

Specific Aim 1: To evaluate the contribution of IAP protein expression to the rituximab/chemotherapy resistance seen in rituximab resistant cell lines

Specific Aim 2: To define the anti-tumor activity of small molecule IAP antagonists in rituximab resistant B-cell lymphoma
Chapter 2 – The X-linked inhibitor of apoptosis protein (XIAP) regulates chemotherapy resistance in rituximab resistant cell line models

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Abstract

The observations that RRCLs have a defective apoptotic response led us to hypothesize that IAP family proteins may be contributing to RRCL chemotherapy resistance. To investigate if IAPs were upregulated in RRCLs we used a both western blot and quantitative PCR systems, and we observed that expression of the IAPs survivin and livin were upregulated in RRCLs. To determine if either of these proteins was contributing to RRCL resistance we used a system of targeted small interfering RNA (siRNA) knockdowns to individually target IAP expression. We measured the anti-tumor effect of each knockdown with an annexin-V apoptosis induction assay. To our surprise, knockdown of survivin or livin had no effect on the resistance to chemotherapy in RRCLs. We also investigated the other IAP family members cIAP1/2, but these two did not appear to be contributing to resistance. In contrast to the other IAP family members investigated, knockdown of XIAP did increase sensitivity to both etoposide and gemcitabine in RRCLs. This result was encouraging, but also surprising because XIAP did not appear to be upregulated in RRCLs. When we investigated how the RRCL Raji 4RH responded to gemcitabine exposure, we discovered that the mitochondria in Raji 4RH do not release normal apoptotic factors when exposed to high dose chemotherapy. The second mitochondria-derived activator of caspases (SMAC) is the normal XIAP antagonist released from mitochondria upon apoptosis induction, but this does not appear to happen in Raji 4RH. Defective SMAC release appears to enhance the anti-apoptotic function of XIAP in RRCLs, even though its expression is unchanged compared to sensitive lymphoma cell lines. To determine if knockdown of XIAP could improve in vivo RRCL chemotherapy responses we created a Raji 4RH cell line with a stable knockdown of XIAP. This Raji 4RH_XIAP KD cell line displayed the same improved response to etoposide and gemcitabine exposure, and our first animal experiment with this cell line demonstrated a survival advantage for the Raji 4RH_XIAP KD animals compared with the Raji 4RH_scramble control animals when each group was given
chemotherapy. Unfortunately, this result was not repeatable. After careful investigation of the Raji 4RH_XIAP KD cell line we observed that, although XIAP was still knocked down, the cells were no longer sensitive to chemotherapy. Additional investigation revealed substantial alterations in the IAP proteins compartment, although the full cause of the new chemotherapy resistance in Raji 4RH_XIAP KD has not yet been determined. In either case, these results demonstrate that XIAP is a key regulator of chemotherapy resistance and cell survival in RRCL models.

Introduction

Previous work done by our group established the importance of the apoptotic regulatory network in rituximab resistant lymphoma models.[23] The identification of the critical loss of Bax and Bak expression in the RRCLs Raji 4RH and RL 4RH, and the subsequent work detailing how restoring Bax expression could trigger spontaneous apoptosis in RRCLs made it very apparent that there was potential utility in exploring additional layers of the apoptotic compartment in RRCL models. A near total loss of both the critical pro-apoptotic Bcl-2 family proteins would almost certainly render a cell resistant many of the normal chemotherapeutic approaches currently in use, as well as novel targeted approaches such as direct inhibition of the anti-apoptotic Bcl-2 family members. Restoration of normal Bax expression itself is not a feasible therapeutic approach; however it did lead our group to hypothesize that there may be other areas of the apoptotic regulatory network that could be a therapeutically targeted. The most initially appealing of these were the inhibitor of apoptosis family proteins. The goals of these studies were both to establish if expression of the IAP family proteins is deregulated in RRCL models, and also to determine if targeting IAPs has any anti-tumor effect.
Materials and Methods

Cell lines
The human lymphoma cell lines used for *in vitro* experiments were purchased from the Leibniz-Institute/German Collection of Microorganisms and Cell Cultures (DSMZ). The rituximab/chemotherapy resistant cell lines Raji 4RH and RL 4RH were created as previously described.[21, 23] All cell lines were maintained in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 5mM HEPES at 37°C, 5%CO2. Cell lines are all maintained in culture for less than 6 months continuously to prevent genetic drift.

Reagent, antibodies, and equipment
Primary western blot antibodies and HRP conjugated secondary antibodies were purchased from Cell Signaling (Danvers, MA). RIPA buffer, trypan blue, and histopaque-1077 were obtained from Sigma-Aldrich Inc. (St Louis, MO). Protease inhibitor cocktail set I and phosphatase inhibitor cocktail set V were purchased from EMD Millipore (Billerica, MA). The Cell Titer-Glo Luminescent Viability Assay and the Caspase-Glo luminescent caspase activity assays were purchased from Promega (Madison, WI). All luminescent assays were read on a Synergy HTX plate reader purchased from Biotek (Winooski, VT). Annexin V-PE/Cy7 and the Sytox Blue DNA stain for apoptosis studies were purchased from Thermo Fisher Scientific. All flow cytometric studies were run on a Becton Dickinson LSR-II flow cytometer at the Roswell Park flow cytometry core facility. Results were processed with FCS express software version 4 purchased from De Novo software of Glendale, CA.
Quantification of IAP protein expression

Base line expression levels of IAP proteins were determined by western blot. Protein lysates were extracted from Raji, Raji 2R, Raji 4RH, RL, and RL 4RH using a RIPA buffer solution containing protease and phosphatase inhibitors added according to manufacturer specifications. Cells were lysed at 4°C for 30 minutes; nuclei and debris were pelleted at 13,000 rpm for 30 minutes in an Eppendorf microcentrifuge. Lysates were made with crude protein extract, 4x laemmli buffer, and deionized water, and then run on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane using the iBLOT system (Invitrogen Technologies, Grand Island, NY). Membranes were blocked for a minimum of 1 hour with 5% milk in tris-buffered saline with tween (TBST) and then incubated at 4oC overnight with antibodies directed against proteins of interest. HRP-conjugated secondary antibody was used for detection with the ECL-Plus enhanced chemiluminescence visualization system (Amersham Life Sciences, Arlington Heights, IL). Experiments were repeated on three separate occasions.

Quantification of IAP gene expression

Quantitative PCR was performed with the TaqMan Cells to Ct one-step kit purchased from Thermo Fisher Scientific, Waltham, MA. TaqMan probes for the Birc5 and Birc7 genes, along with a control TaqMan probe for 18s ribosomal RNA, were purchased from Thermo Fisher Scientific, Waltham, MA. All samples were run on a QuantStudio 6 unit, and the data presented is the combined results of 3 experiments analyzed together using QuantStudio real time PCR software (Thermo Fisher Scientific, Waltham, MA).

IAP knockdown studies

XIAP, livin, survivn and cIAP1/2 siRNA knockdowns were performed with OnTarget-Plus siRNA smart pools for each on the specified IAPs. Controls for all siRNA transient knockdown studies were the OnTarget-plus siRNA non-targeting smart pool. All siRNAs were purchased from
Dharmacon (Lafayette, CO). All siRNA transfections were performed on an Amaxa Nucleofector II electroporation unit purchased from Lonza Switzerland. All electroporation reactions were performed with an Amaxa cell line kit V. All knockdowns were confirmed through western blot. Images presented are from one experiment, but representative of all three.

*In vitro* apoptosis induction studies

Apoptosis induction was measured with Annexin V / Sytox Blue staining after 48 hours of exposure to the indicated treatment and concentration. Experiments were performed in triplicate. The results presented are graphs of one experiment, which is representative of all three.

**Caspase activity studies**

Cells for caspase activity studies were pretreated for 36 or 48 hours with either chemotherapy at indicated concentrations or media control. Caspase activity was measured in 384 well plates loaded with $2.5 \times 10^3$ cells per well. Caspase activity was measured with the Promega luminescent Caspase-Glo assay. Specific assays were used to measure the activity of caspases 3 and 7 together, caspase 6 individually, and caspase 2 individually. Experiments were performed in triplicate.

**Luminescent *in vivo* tumor models**

Raji and Raji 4RH cell stably transfected with a luciferase reporter vector were a generous gift from the laboratory of Mitchell Cairo at the New York Medical College of Valhalla, New York.[62] Animals were inoculated with $10 \times 10^6$ Raji 4RH_Luc cells. R-ICE chemotherapy consisted of rituximab (30mg/kg i.v.), ifosfamide (300mg/kg i.p. with MESNA rescue also (300mg/kg i.p.), carboplatin (40mg/kg i.v.), and etoposide (12mg/kg i.v.). Animals were given chemotherapy 7 days after tumor implantation, and monitored weekly by whole body luminescent scan.
Statistical analyses

Statistical significance for the *ex vivo* patient sample studies was determined through a one directional ANOVA test performed with SPSS Statistics software version 21 purchased from IBM Corporation, NY.

Results

Expression levels of the IAPs survivin and livin are increased in RRCLs

Initially we used a series of western blots to investigate which, if any, alterations in IAP expression there may be between RRCLs and sensitive parent cell lines. The most obvious changes we observed were in the expression of livin and survivin. Although survivin and livin appear to be upregulated in RRCLs, other members of the IAP family are more equivalently expressed. XIAP and cIAP1 were slightly more expressed in the RRCLs, while cIAP2 expression levels were essentially equal across all cell lines investigated. (Figure 2.1a.)

Although we didn’t observe upregulation in all IAP family members, our initial observations with survivin and livin convinced us to investigate IAP upregulation further through experiments to

![Western blot analysis](image1.png)

![Quantitative PCR results](image2.png)
confirm if the changes in survivin and livin protein levels correlated with an increase in Birc5 (survivin) and Birc7 (livin) mRNA levels. We performed a Taqman quantitative PCR reaction, which revealed increased mRNA levels for both Birc5 (survivin) and Birc7 (livin) in RRCLs compared to sensitive parent cell lines. (Figure 2.1b.) While Birc7 was expressed in the RL cell line, it was not quantifiably expressed in the Raji cell line. The observed increase in survivin and livin expression was enough to convince us to investigate if IAPs are contributing to the chemotherapy resistance observed in rituximab resistant cell lines.

**Survivin and livin do not affect RRCL chemotherapy resistance**

To determine if survivin and livin were actually contributing to RRCL chemotherapy resistance, or if these were just bystander alterations we used a series of targeted siRNA (small interfering RNA) knockdowns to cleanly deplete either survivin or livin. We then tested RRCLs transfected with the targeting siRNA, or a non-targeting scramble control siRNA, with the clinically used anti-lymphoma chemotherapy agents vincristine, etoposide, and carboplatin at therapeutically relevant doses. Raji 4RH cells transfected with livin targeting siRNA (livin knockdown) responded to chemotherapy exposure in an almost identical fashion when compared to Raji 4RH cells transfected with a non-targeting scramble siRNA. (Figure 2.2a, 2.2b) This was measured through Annexin-V staining. The induction of apoptosis through chemotherapy exposure was negligible in both Raji 4RH cell transfected with a non-targeting scramble siRNA, or Raji 4RH cells transfected with a livin targeting siRNA. c) The knockdown of livin was confirmed through western blot.

![Figure 2.2 The knockdown of livin in Raji 4RH does not affect chemotherapy sensitivity](image)
staining (with Sytox Blue as a DNA stain) to indicate the rates of apoptosis induction in treated cells. The knockdown of livin was confirmed through western blot 48 hours after transfection, but before incubation with chemotherapy. (Figure 2.2c) To investigate the impact of survivin expression on chemotherapy resistance in Raji 4RH we used a similar siRNA knockdown electroporation approach. Raji and Raji 4RH cells were transfected with either a non-targeting scramble control, or a survivin targeting siRNA. As observed with the knockdown of livin, a knockdown of survivin did not alter the rates of apoptosis induction observed in cells treated with either etoposide, gemcitabine, or vincristine. (Figure 2.3a, 2.3b) Because survivin expression can be induced by cell stress we took the additional step of verifying the integrity of the survivin knockdown in each of the treated cell groups. The knockdown of survivin remained durable even following 48 hours of incubation with chemotherapy agents. (Figure 2.3c) Exposure to 50uM etoposide did produce a small, but detectable, increase in survivin expression in both the cells with a survivin knockdown, as well as the scramble control cells. It is worth noting that, even with the slight increase in survivin expression observed in the survivin knockdown cells treated with etoposide, the overall survivin expression in these cells was well below the survivin expression level in Raji 4RH cells electroporated with the scramble control.
**clAP1/2 do not affect RRCL chemotherapy resistance**

Although our initial experiments with the knockdown of survivin and livin in Raji 4RH demonstrated no benefit, we felt it was necessary to investigate the IAP family more closely in RRCLs before we could conclude that IAPs are truly not important in RRCL biology. Two of the most well studied members of the IAP family are clAP1 and clAP2. Each of these proteins have been shown to contribute to apoptotic resistance in numerous experimental models,[38] and we hypothesized that either clAP1, clAP2, or both together may be exerting an anti-apoptotic effect in RRCLs. To test this we used the same siRNA transfection system we employed in our studies of survivin and livin. Raji 4RH cells were transfected with siRNA targeting clAP1, clAP2, or a mixture of both siRNA constructs. Once again a non-targeting scramble siRNA served as a control. All cells (both knockdown and scramble control cells) were incubated with chemotherapy at the indicated doses for 48 hours.

Figure 2.4 The knockdown of clAP1/2 in Raji 4RH does not affect chemotherapy sensitivity

a), b), c), d) The rates of apoptosis were measured through annexin-V staining. The induction of apoptosis through chemotherapy exposure was negligible in Raji 4RH cell transfected with a non-targeting scramble siRNA, either clAP1 or clAP2 siRNAs, or a clAP1/2 combination knockdown. c) The knockdown of both clAP1/2 was confirmed through western blot 96 hours after transfection, and 48 hours after chemotherapy exposure.

Once again we measured the induction of apoptosis with annexin-V staining. As with our survivin and livin knockdown experiments, we observed no change in the rates of apoptosis
induction when cIAP1/2 were depleted in Raji 4RH. (Figure 2.4a, 2.4b, 2.4c, 2.4d) We confirmed the durability of the knockdowns with a series of western blots after the cells had been incubated with chemotherapy agents for 48 hours. (Figure 2.4e) The knockdown of cIAP1 alone was complete and durable, even after chemotherapy exposure. The knockdown of cIAP2 was mostly durable, except when cells were exposed to 50uM gemcitabine. Exposure to gemcitabine at this concentration appeared to partially reverse the siRNA knockdown. We hypothesize this is through an intrinsic cellular response to gemcitabine induced stress, although we are not certain about this. In contrast to gemcitabine, etoposide exposure at 50uM appeared to sharply decrease expression of cIAP1 and cIAP2 regardless of which siRNA the cells had been transfected with. Even Raji 4RH cell transfected with the non-targeting control siRNA had decreased levels of cIAP1 and cIAP2 after etoposide exposure. The knockdown of cIAP1 and cIAP2 together was less effective than either single knockdown. Raji 4RH control cells transfected with cIAP1 and cIAP2 targeting siRNA still retained detectable levels of both proteins, although levels were less than those seen in Raji 4RH cells transfected with non-targeting siRNA. Because the induction of apoptosis in response to chemotherapy exposure was quite low regardless of cIAP1/2 depletion status, or the effect of chemotherapy alone on cIAP1/2 expression, we were forced to concluded that neither cIAP1 or cIAP2 substantially contributes to chemotherapy resistance in RRCLs.
**XIAP regulates chemotherapy response and survival in RRCLs**

The negative results we observed with knockdowns of livin, survivn, and cIAP1/2 we a surprising result the forced us to revisit our hypothesis that IAPs were regulating cell survival in RRCLs. If survivin, livin, and both the cIAP1/2 proteins were not contributing to chemotherapy resistance, we hypothesized that only one other IAP family member was likely to be actively contributing to RRCL resistance. Given its role as a direct caspase inhibitor we decided to investigate how XIAP was impacting chemotherapy resistance in RRCLs. Once again we employed the same transient siRNA knockdown strategy to knockdown XIAP in the Raji 4RH and Raji cell lines. The knockdown of XIAP in each cell line was confirmed through western blot. (Figure 2.5a) The knockdown of XIAP was nearly complete in both the Raji and Raji 4RH cell lines. One difference we observed on the western blots between Raji and Raji 4RH was the degree of PARP cleavage (an indicator of apoptosis induction) in cells transfected with the XIAP targeting siRNA. Raji with an XIAP knockdown showed no PARP cleavage, but the Raji 4RH cell line displayed considerable PARP cleavage, indicating to us that XIAP may be specifically important for Raji 4RH cell survival.

Figure 2.5 The knockdown of XIAP improves chemotherapy response in Raji 4RH

<table>
<thead>
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<th>a.</th>
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<td>Control</td>
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<tr>
<td>XIAP KD</td>
<td>XIAP KD</td>
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<tr>
<td>XIAP</td>
<td>+</td>
</tr>
<tr>
<td>PARP</td>
<td>+</td>
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<td>B-Actin</td>
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a) XIAP knockdown was confirmed by western blot. In the Raji 4RH cell line loss of XIAP increased the level of cleaved PARP, although no apoptosis was observed through Annexin V/Sytox Blue staining. b) siRNA knockdown of XIAP alone was not cytotoxic in Raji or Raji 4RH cell lines. Rates of apoptosis were similar to scramble controls. c) XIAP knockdown in combination with 50uM Etoposide, or d) 50uM Gemcitabine exposure for 48 hours significantly increased the rate of apoptosis in Raji 4RH cell, but had no effect on Raji cells. * = p<0.05
Although we were intrigued by our observations that XIAP knockdown was a strong stressor in Raji 4RH cells, we felt it was important to determine if XIAP knockdown could actually alter the sensitivity of Raji 4RH cells to chemotherapy agents. To do this we incubated both Raji and Raji 4RH cells with clinically relevant doses of five commonly used anti-lymphoma chemotherapies. Both etoposide and gemcitabine increased the rates of apoptosis in Raji 4RH XIAP knockdown cells compared to Raji 4RH scramble control cells. Etoposide, in particular, increased the levels of Raji 4RH apoptosis to approximately the same levels seen in the Raji cell line exposed to etoposide at the same dose. (Figure 2.5b, 2.5c, 2.5d) No differences in the rates of apoptosis were observed in Raji cells with an XIAP knockdown versus a Raji cells with a scramble control transfection. This indicated to us that XIAP is contributing to chemotherapy resistance in Raji 4RH cells, and that XIAP is specifically important in a rituximab resistant model, and not simply a general pro-survival protein important in all contexts.

To determine if the results we observed in Raji 4RH would hold for our other RRCL model RL 4RH we used the same siRNA transfection system. When we verified the knockdown of XIAP in RL and RL 4RH cells we observed that the knockdown was durable, but also that loss of XIAP appeared to be even more cytotoxic in the RL 4RH cells line. (Figure 2.6a) We observed...
significant PARP cleavage, and importantly, a decrease in the amount of full length PARP in RL 4RH XIAP knockdown cells. In Raji 4RH XIAP knockdown cells we observed an increase in the cleavage fragment of PARP, but these cells still had approximately equal levels of full length PARP compared to Raji 4RH scramble control cells. The decrease in full length PARP observed in RL 4RH cells when XIAP was knocked down, along with the accumulation of the PARP cleavage fragment, indicated the these cells were fully undergoing apoptosis, and not just highly stressed. We performed additional annexin-V/sytox blue staining on these cells to confirm the induction of apoptosis, and we observed an increase in apoptosis in RL 4RH cells when XIAP was depleted. (Figure 2.6b) We also observed no change in the apoptotic rates of RL cells regardless of XIAP knockdown status. These results were very exciting to us, and demonstrated that XIAP was regulating cell survival in RRCL models.

**RRCLs exhibit impaired MOMP induction**

The transient knockdown experiments targeting XIAP in Raji 4RH and RL 4RH indicate that XIAP is specifically important in the RRCL models. Although loss of XIAP increased the activity of chemotherapy agents in Raji 4RH and RL 4RH, it had little to no effect in the parental Raji and RL cell lines. To determine why XIAP is selectively important in RRCL models we investigated how the mitochondrial responses to chemotherapy (50uM gemcitabine) differed in both the Raji and Raji 4RH cell lines. We found that 28 hours of gemcitabine exposure was the ideal duration to induce the first stages of apoptosis, but not long enough to allow for the broader proteomic degradation which occurs during later stage apoptosis. To investigate the release of apoptotic factors from the mitochondria into the cytoplasm after chemotherapy exposure we isolated mitochondrial and cytoplasmic fractions from both gemcitabine treated, and control cells. Exposure to gemcitabine for 28 hours resulted in an accumulation of cytochrome C in the cytoplasm of Raji cells, which was mirrored by an observed decrease in cytochrome c in the mitochondrial fraction taken from the same Raji cells (Figure 2.7a, 2.7b). In
addition we observed a similar pattern of effect with the endogenous XIAP inhibitor SMAC, which was increased in the cytoplasm of Raji cells following 28 hour gemcitabine exposure, and decreased in the mitochondrial fraction. We also investigated another endogenous XIAP inhibitor HTRA2, also referred to as Omi, which was faintly observable in the mitochondrial fractions of untreated Raji cells. The presence of these factors in the cytoplasm and their corresponding decrease in the mitochondrial fractions following gemcitabine exposure is consistent loss of mitochondrial outer membrane integrity (MOMP), and the initiation of apoptosis, which was verified through western blot for PARP cleavage in Raji.

When we performed the same series of experiments with the Raji 4RH cell line we observed

**Figure 2.7 The release of the apoptotic factors is blocked in RRCLs**

<table>
<thead>
<tr>
<th>Cytoplasmic Fraction</th>
<th>Mitochondrial Fraction</th>
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<tr>
<td>a.</td>
<td>b.</td>
</tr>
<tr>
<td>Gemcitabine 50µM</td>
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</tr>
<tr>
<td>Raji 4RH</td>
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<tr>
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<td>-</td>
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<tr>
<td>HTRA2/Omi</td>
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<tr>
<td>Actin</td>
<td>-</td>
</tr>
<tr>
<td>PARP</td>
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a) Cytoplasmic fractions from Raji and Raji 4RH cells treated with gemcitabine for 28 hours display PARP cleavage in the Raji cell line, but not in the Raji 4RH cell line. Raji cells also display an increase in the cytoplasmic levels of cytochrome c and SMAC were observed. No cytochrome c was observed in the cytoplasmic fractions of Raji 4RH, and the level of SMAC decreased after exposure to gemcitabine. b) Mitochondria isolated from treated and control cells mirror the results observed in the cytoplasmic fractions. Levels of cytochrome c and SMAC are lower in Raji cells after gemcitabine exposure, but appear to increase in Raji 4RH cells. c) Baseline expression of procaspase 3 and procaspase 7 in Raji 4RH was nearly undetectable compared to the sensitive cell line Raji. XIAP baseline expression appears to be unchanged between Raji and Raji 4RH.

several key differences. Most importantly, cytochrome C was not released following gemcitabine exposure, and even appeared to be more strongly retained in the mitochondria of
Raji 4RH cells. SMAC levels in the cytoplasmic fractions also appeared to decrease following gemcitabine exposure, which corresponded to an observed increase in the SMAC present in the respective mitochondrial fraction. Somewhat paradoxically, SAMC was detectable in the cytoplasm of Raji 4RH cells in the absence of any chemotherapy exposure, which suggests that additional anti-apoptotic events may be contributing to Raji 4RH chemotherapy resistance. These results indicate that the early mitochondrial apoptotic response to chemotherapy is blocked in the Raji 4RH cell line, which results in decreased release of the endogenous inhibitors of XIAP.

Our observations that SAMC is present in the cytoplasm of Raji 4RH cells at baseline suggests a model system where SMAC is of decreased overall importance in the maintenance of apoptotic hemostasis in RRCLs. This prompted us to investigate the expression levels of the primary effector caspases 3 and 7, which are released from XIAP after SMAC release from the mitochondria. When we examined the baseline expression of full length pro-caspase 3 and pro-caspase 7 we observed that, in comparison to the parental Raji cell line, Raji 4RH cells expressed almost no detectable pro-caspase 3 or pro-caspase 7 (Figure 2.7c).
XIAP knockdown promotes caspase dependent cell death in Raji 4RH

The observation that Raji 4RH cells have decreased pro-caspase 3/7 expression led us to hypothesize that depletion of XIAP in Raji 4RH promotes cell death through a caspase 3/7 independent mechanism.

To investigate this we use a transient siRNA knockdown system to decrease XIAP levels in the RRCL cell lines with a nonspecific scramble siRNA serving as a control.

The knockdown of XIAP was confirmed in Raji 4RH and RL 4RH by western blot. (Figure 2.8a, 2.8b) When exposed to 20uM etoposide for 36 hours Raji 4RH XIAP_KD cells were significantly less viable than Raji 4RH scramble controls (p<0.000001), which was fully reversed by the addition of QVD-Oph. A spike in caspase 3/7 activity is seen in both the Raji scramble and Raji XIAP knockdown cells treated with 20uM etoposide. Raji XIAP KD cells did display a stronger response to 20uM etoposide compared to Raji scramble cells (p=0.044) 

2.9a). This increase in cell death corresponded with statistically significant increase in caspase 3/7 activity in Raji 4RH XIAP_KD cells exposed to etoposide, compared to Raji

2.9a).
4RH_scramble cells treated with etoposide (p=0.000065) (Figure 2.9b). To determine if the observed drop in viability was caspase dependent, or merely coincidental with caspase activation, we included the pan-caspase inhibitor QVD-OPh (at a 10uM concentration) with the etoposide treatment to block caspase activity. Caspase inhibition was sufficient to rescue Raji 4RH XIAP_KD cells treated with etoposide, and eliminated the anti-tumor effect of XIAP knockdown. This pattern activity was also observed when cells were exposed to etoposide for 48 hours (Figure 2.9c,2.9d). Although we observed less difference in etoposide induced caspase 3/7 activity between Raji 4RH scramble and Raji 4RH XIAP_KD cells, it was still statistically significant (p=0.002). Although the difference is caspase 3/7 activity was lower at 48 hours, the impact of XIAP knockdown on cell viability appeared to increase. Raji 4RH XIAP_KD cells were significantly less viable (p=0.000245) after 48 hours of exposure to 20uM etoposide when compared to scramble controls. Once again this increase in etoposide sensitivity was blocked by QVD-OPh, indicating that the anti-tumor effect of XIAP knockdown is caspase 3/7 dependent. The increased caspase activity at 36 hours, and the decreased viability observed at...
48 hours of incubation are consistent with the generally accepted sequence of events in apoptosis where caspase activation precedes the full shutdown of cell functioning. Caspases 3 and 7 are regarded as the primary apoptotic effector caspases, but there are additional caspases which have also been reported to drive apoptosis under certain conditions, specifically caspases 2 and 6.\cite{63, 64} To determine if either of these two caspases were contributing to the observed effect of XIAP knockdown we used two specific caspase activity assays, similar to the caspase 3/7 activity assay. We observed no substantial differences in the activity of either caspase 2 or caspase 6 between Raji 4RH scramble and Raji 4RH XIAP\_KD cells at both the 36 and 48 hour time points. (Figure 2.9a, 2.9b, 2.9c, 2.9d) This result indicated to us that XIAP knockdown in Raji 4RH cells was increasing etoposide induced apoptosis in a caspase 3/7 dependent manner. The knockdown of XIAP was confirmed in Raji 4RH and RL 4RH by western blot. (Figure 2.10a, 2.10b)

**Stable knockdown of XIAP increases the cytotoxic effect of chemotherapy exposure in Raji 4RH cells**

One complication of the electroporation procedure we used to perform our transient knockdowns of XIAP is that the electroporation procedure can be very stressful to the cells being transfected. Additionally, siRNA knockdowns will remain stable in cells for a limited amount, which limits the types of experiments that can be performed. To address these two limitations of our transient siRNA knockdown system we worked with the Roswell Park genetics core facility to create a Raji 4RH cell line stably transfected with shRNA (short hairpin RNA) expression vector designed to target XIAP. The Raji 4RH cell line we used was also stably
transfected with a construct to constitutively express luciferase to facilitate tracking of engraftment during in vivo experiments. We chose two similar shRNA sequences, both designed to target XIAP. Each sequence was designed to target portions of the XIAP mRNA the were also targeted by the siRNA pool we employed in our transient knockdown experiments in an attempt to mirror the transient knockdown experiments as closely as possible. After transfection and selection of the two stable Raji 4RH XIAP knockdown cell lines (Raji 4RH_XIAP KD) we analyzed XIAP expression levels to determine which cell line had a more complete knockdown. (Figure 2.11a) We selected the cell line transfected with the V2LHS_94579 construct based on the higher degree of XIAP knockdown compared to a Raji 4RH cell line transfected with a non-targeting scramble control construct (Raji 4RH_scramble). Knockdown of XIAP in Raji 4RH was did not increase the rate of apoptosis on its own, as measure by annexin V staining. (Figure 2.11b) This result confirmed that loss of XIAP is tolerable in the Raji 4RH line, although still presumably stressful to the cells. When the Raji 4RH XIAP_KD cell line was exposed to either etoposide or gemcitabine (both at 50uM) we observed a much stronger apoptotic response compared to the normal Raji 4RH cell line, and the Raji 4RH_scramble control line. (Figure 2.12a, 2.12b) Apoptotic rates in the Raji 4RH XIAP_KD line were similar to those

Figure 2.11 Stable knockdown of XIAP in Raji 4RH was not cytotoxic

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<tr>
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<tr>
<td>XIAP_KD1</td>
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a) Two stable shRNA knockdowns of XIAP were generated in Raji 4RH. The clone with the second construct was selected for further studies because of the lower XIAP expression levels it displayed. b) Stable knockdown of XIAP did not change the rate of rate of apoptosis without chemotherapy exposure.
observed in the rituximab sensitive parental cell line Raji. Although loss of XIAP enhanced the activity of etoposide and gemcitabine, we observed no increase in response to vincristine or carboplatin in the Raji 4RH XIAP_KD cell line. (Figure 2.12c, 2.12d) These results support our earlier findings with transient XIAP knockdown models, and indicate that the electroporation itself was not substantially affecting the results of our XIAP knockdown studies.

Loss XIAP in Raji 4RH cells can increase in vivo responses to chemotherapy.

To investigate if XIAP knockdown could increase Raji 4RH chemotherapy sensitivity in vivo we implanted SCID mice with the Raji 4RH_XIAP KD cell line, and the Raji 4RH_scramble cell line as a control. Animals were each inoculated with 10x10^6 cells by tail vein injection to facilitate disseminated tumor engraftment. After allowing 7 days for tumor engraftment, animals were divided into treatment and control groups. We chose to treat animals with a combination of rituximab, etoposide, ifosfamide, and carboplatin to mimic the R-ICE salvage chemotherapy regimen commonly used in relapse/refractory aggressive lymphoma patients. Initially, animals inoculated with the Raji 4RH_XIAP KD cell line appeared to respond better to R-ICE than animals inoculated with the Raji 4RH_scramble cell line. (Figure 2.13a) Although the first in vivo experiment with the Raji 4RH_XIAP KD cells indicated that knockdown of XIAP could increase survival, these results could not be replicated. In subsequent animal experiments we observed no survival advantage in animals inoculated with the Raji 4RH_XIAP KD cell line.
This result was quite unexpected, and it prompted us to investigate if the Raji 4RH_XIAP KD cell line was still as sensitive to chemotherapy as it initially had been. When we compared the effect of 48 hours of exposure to etoposide at 20μM in both the Raji 4RH_scramble and Raji 4RH_XIAP KD cell lines, we observed no increase in apoptosis in the Raji 4RH_XIAP KD samples. (Figure 2.13b) These results lead us to hypothesize that the shRNA knockdown of XIAP was not as stable as we initially believed.

To determine if the knockdown of XIAP in Raji 4RH was stable long term we compared protein lysates from Raji 4RH_XIAP KD cells, which had been in culture for approximately 3 months, with protein lysates made from Raji 4RH_scramble cells immediately after the initial transfection and selection process. We observed that the knockdown of XIAP was indeed
stable long term. XIAP protein levels were unchanged between the initial Raji 4RH_XIAP KD cells and the Raji 4RH_XIAP KD resistant cells. Both of these cell lines had XIAP expression levels well below levels observed in both Raji and the original Raji 4RH cell lines. (Figure 2.13c) Although the knockdown of XIAP was unchanged in the Raji 4RH_XIAP KD resistant cells, other parts of the apoptotic compartment showed alterations when compared with the initial Raji 4RH_XIAP KD cell line. The levels of procaspase 3 and procaspase 7 (the zymogen forms of caspases 3 and 7) were substantially increased in the resistant Raji 4RH_XIAP KD cells, indicating that some other alteration removed the selective pressure against caspase 3/7 expression in these cells. In addition, the expression the IAP livin appeared to steadily increase in both of the Raji 4RH_XIAP KD cell lines. These results suggested to us that loss of XIAP expression in the Raji 4RH cells results in a substantial selective pressure, which promotes additional alterations in the apoptotic compartment. The exact mechanism of XIAP knockdown resistance in the Raji 4RH_XIAP KD cells has yet to be identified.

Discussion

The observation that loss of XIAP in RRCLs increases the cytotoxic effect of etoposide and gemcitabine confirms our hypothesis that IAP family proteins are promoting tumor cells survival in RRCL models. In addition, our findings indicate that RRCLs are specifically sensitive to loss of XIAP, rather than loss of cIAP1/2, which supports a model where caspase sequestration is more important than other IAP functions, such as ubiquitin conjugation. This model is further supported by our data indicating that the cytotoxic effect of XIAP knockdown in RRCLs is caspase dependent. These points are of particular importance, given that most of the therapeutic efforts to target IAPs to date have focused on cIAP1/2, rather than XIAP.[41] Despite significant sequence similarity to several other IAP family proteins (cIAP1 and cIAP2) XIAP is unique in its ability to stably bind and sequester the active forms of caspases 3 and 7.[65] This action happens at the XIAP BIR2 domain (short for baculovirus IAP repeat 2), which
is distinct and separate from the caspase 9 binding that occurs at the XIAP BIR3 domain.[66] This attribute makes it an attractive choice for targeted inhibition with small molecules, and our findings suggest that rituximab relapse/refractory lymphomas may be sensitive to XIAP mono-inhibition. Given the synergistic toxicity seen in animals with combined knockout of XIAP and cIAP1, selective targeting of XIAP may offer a better side effect profile.[67]

The enhanced cytotoxic effect of XIAP inhibition with cIAP1 inhibition is an important consideration when XIAP inhibition is combined with chemotherapy exposure. Our results indicate that exposure to etoposide and carfilzomib can decrease the levels of cIAP1 in the cytoplasm, which may be triggering spontaneous cell death when combined with XIAP gene expression knockdown. Exposure to etoposide at a high dose (100uM) has been demonstrated to induce the formation of an alternative cell death initiation complex termed the ripoptosome. This large (~2MDa) multi-protein complex involves the RIPK1 kinase, and it has been shown to function as an activator of caspase 8, as well as an activator of caspase independent programmed cell necrosis.[68] If the ripoptosome complex was controlling XIAP knockdown associated cell death in Raji 4RH cells we would expect to observe robust caspase independent cell death when XIAP knockdown was combined with etoposide exposure. Our results indicate that activity caspases 3 and 7 is required for XIAP knockdown mediated cell death, and that caspase inhibition effectively reverses that effect of XIAP knockdown in Raji 4RH cells; however, some degree of ripoptosome formation following XIAP knockdown may be triggering the activation of caspases 3/7 through caspase 8 activation. To fully investigate this we would need to conduct additional studies with RIPK1 knockout RRCL models.

The emergence of subsequent chemotherapy resistance mutations in the Raji 4RH cell line to compensate for XIAP knockdown represents an unfortunate setback for this body of work; however, it also presents an important opportunity to investigate how the IAP family of proteins interact with the broader cell death regulatory systems within a cell. The observations that
levels of procaspase 3 and procaspase 7 increase in the Raji 4RH XIAP_KD resistant cell line indicate that the mechanism for XIAP knockdown adaptation likely involves some block to upstream activation of the caspase cascade. This may be something as simple as loss of caspase 8 expression; however, it may also involve broader changes to the cell death response pathways. We are currently discussing how best to unravel the potentially complex series of changes in these cells, but any solution will probably require a systems biology based approach with full mRNA expression sequencing, along with proteomic analysis.

The clinical significance of XIAP expression in tumors has not yet been uniformly established. To date the impact of XIAP expression on lymphoma biology has not been fully established, and most of the attempts to target XIAP in hematological malignancies have preclinical studies. More is known about the role of XIAP solid tumors, such as pancreatic carcinoma where high expression of XIAP, as determined by immunohistochemistry staining, was associated with a statistically significant decrease in survival. This pattern of XIAP association with decreased survival was also observed in esophageal carcinoma [69]; however the inverse was observed in prostate cancer where XIAP expression appears to correlate with improved overall survival.[70] The discrepancy in these results is probably due as much to the complexity of the apoptotic regulatory network as it is to differences between tumor types. The full impact of XIAP on cell resistance is difficult to predict based on XIAP expression levels alone. Expression of XIAP is not upregulated in RRCLs; however, the cytoplasmic levels of other XIAP interacting apoptotic factors, such as SMAC and caspases 3/7, do appear to be deregulated in RRCLs. We hypothesize that the end result of all these alterations is the enhanced importance of XIAP in tumor cell survival; however, fully exploring this hypothesis in patient tissue samples would require investigating the expression levels and subcellular localization of many apoptotic factors. Hopefully more complex retrospective studies of the entire apoptotic compartment in tumor
biopsy samples will clarify the clinical role of XIAP in regulating disease progression and chemotherapy resistance.
Chapter 3 – Small molecule IAP inhibitors exhibit anti-tumor effect B-cell lymphoma models, including rituximab resistant lymphoma models

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Abstract

Our results confirming that XIAP is a keystone regulator of resistance in RRCLs prompted us to hypothesize that small molecule inhibitors of XIAP could overcome the resistant phenotype of RRCLs, and increase chemotherapy induced apoptosis in RRCLs. To test this hypothesis we obtained two IAP inhibitors, LCL-161 and BMT-062789. We used a luminescent ATP quantification assays to measure the anti-tumor effect of both LCL-161 and BMT-062789 in a panel of lymphoma cell lines, including RRCLs. LCL-161 has single agent IC50 concentrations in the 20μM to 50μM range. We used western blot to investigate the how LCL-161 exposure impacted the expression levels of IAPs in RRCLs. Both of the cIAP1/2 proteins were depleted at even a 1μM concentration of LCL-161 in the Raji 4RH cell line, but only cIAP1 was effected in the RL 4RH cell line. In both cell lines the expression of survivin and livin was also decreased at higher concentrations of LCL-161. To determine if LCL-161 could enhance chemotherapy activity in RRCLs we cultured Raji 4RH cells with LCL-161 and various chemotherapy agents for 48 hours. We observed that LCL-161 synergistically enhanced the anti-tumor activity of etoposide, gemcitabine, and vincristine in the Raji 4RH cell line, with less synergistic responses seen in the Raji cell line. LCL-161 also synergistically enhanced the anti-tumor effect of the proteasome inhibitor carfilzomib in both Raji and Raji 4RH cell lines. To quantify the in vivo anti-tumor effect of LCL-161 we used a SCID mouse model implanted with a disseminated Raji 4RH tumor model. LCL-161 combined with chemotherapy produced a statistically significant increase long term survival compared to chemotherapy alone. We also investigated the anti-tumor effect of LCL-161 in ex vivo lymphoma patient samples, and we observed that LCL-161 could enhance the anti-tumor effect of carfilzomib in tissue samples from patients with a variety of lymphomas, including therapy relapse lymphomas.

Although LCL-161 demonstrated good anti-lymphoma activity, we hypothesized that a more selective XIAP inhibitor, such as BMT-062789, would have better activity in RRCLs. Single
agent $IC_{50}$ values for BMT-062789 were in the low micromolar range for most cell lines tested, but not the Raji 4RH cell line. To determine if the combination of BMT-062789 and chemotherapy was more active than either alone we incubated RRCLs with BMT-062789 and etoposide. The rates of apoptosis (measured by annexin-V positivity) increased when BMT-062789 and etoposide were combined in both the Raji 4RH and RL 4RH cell lines. To investigate the activity of BMT-062789 in a more clinically relevant model, we exposed *ex vivo* lymphoma patient tissue samples to 0.3uM and 3uM concentrations of BMT-062789. The 3uM concentration of BMT-062789 was active in all *ex vivo* samples tested, and resulted in almost total tumor cell death. These results support our hypothesis that targeted XIAP inhibition with small molecule compounds is an effective anti-lymphoma strategy. In addition, selective XIAP inhibition appear to be a more effective anti-tumor approach in RRCL models.

**Introduction**

The results of the siRNA knockdown experiments targeting IAPs in RRCLs indicated to us that XIAP was influencing the chemotherapy resistance of RRCLs. We hypothesized that targeting XIAP would have an anti-tumor effect in RRCLs, but we also recognized the need to explore more translationally realistic approaches to targeting XIAP. Although some success has been achieved with XIAP anti-sense RNA therapy, the most effective approach for pharmacologically targeting IAPs involves small molecule IAP inhibitors (SMAC mimetics). As a continuation of our past work with the BH3 mimetic obatoclax, which indicated that small molecule agents targeting the anti-apoptotic protein network have activity in RRCLs, we wanted to investigate if IAP inhibitors were active as well. IAP inhibitors have been studied somewhat intensively at this point in preclinical model systems, but clinical trials with these compounds are few and far between and the clinical effectiveness of these compounds is still being investigated.
Small molecule IAP inhibitors are not as specific as targeted siRNA knockdowns, and even the most selective agents will interact with more than one IAP at most IC$_{50}$ doses. Even with these limitations in mind, we concluded that a small molecule IAP inhibitors presented the only translationally viable approach to target XIAP in RRCLs. The two IAP inhibitors we selected for our studies were LCL-161 (a monomeric SMAC mimetic) and BMT-062789. LCL-161 is a conventional SMAC mimetic with binding affinity for the BIR3 (caspase 9 binding) domain of cIAP1/2 and XIAP.[41] BMT-062789, on the other hand, is a more complex molecule with binding affinities for both the caspase binding domains, and selectivity for XIAP.

**Materials and Methods**

**Primary tumor cells**

Neoplastic B-cells were isolated from pre-treatment biopsy tissue obtained from patients with B-cell NHL receiving therapy at Roswell Park Cancer Institute (RPCI) as previously described. Samples from patient biopsy specimens were procured under Institutional Review Board (IRB) RPCI protocols I42804 and I42904. Tissue specimens were placed in PBS-containing collagenase type IV (1mg/ml; Sigma-Aldrich, St. Louis, MO) and incubated for 15 minutes at 37°C, followed by manual agitation for five minutes. Next, samples were diluted with RPMI 1640-containing 10% fetal bovine serum (FBS) and the cell suspension filtered through a 100µm cell strainer to remove large clumps. Lymphocytes were enriched by density centrifugation. B-cells were then isolated from enriched lymphocytes by MACS separation using a human B-cell Isolation Kit II (Miltenyi Biotec, Gladbach, Germany).
Reagent and antibodies

LCL-161 was provided by Novartis AG (Basel, Switzerland). BMT-062789 was obtained from Bristol-Myers Squibb. Rituximab, Gemcitabine, Vinorelbine, and Carfilzomib for experiments were obtained from the RPCI Pharmacy.

All reagents were used at the doses indicated.

Primary western blot antibodies against human IAPs (XIAP, livin, cIAP1/2, and Bruce) were purchased from Cell Signaling (Danvers, MA), along with antibodies against β-actin and poly ADP-ribose polymerase (PARP). Horse radish peroxidase (HRP) conjugated secondary antibodies were also obtained from Cell Signaling. RIPA buffer, trypan blue, and histopaque-1077 were obtained from Sigma-Aldrich Inc. (St Louis, MO). Protease inhibitor cocktail set I and phosphatase inhibitor cocktail set V were purchased from EMD Millipore (Billerica, MA). Cell Titer-Glo Luminescent Viability Assay reagent was purchased from Promega (Madison, WI).

In vitro studies

In order to determine the biologically active dose of LCL-161, a panel of B-NHL cell lines were plated at concentration of 2.5x10^5, and exposed to escalating doses of LCL-161, or vehicle control (Dimethyl sulfoxide [DMSO] 0.001%) for 48 hours. Changes in cell viability were determined by measuring changes in adenosine triphosphate (ATP) content using the Cell Titer-Glo Luminescent Viability Assay reagent (Promega, Madison, WI). Viability of treatment groups was defined as the luminescence reading relative to control group (DMSO exposed). Experiments were performed in triplicates. The half maximal inhibitory concentration (IC_{50}) concentrations were calculated with Prism 6.04 software (GraphPad, La Jolla, CA). Caspase activation studies were performed with the ApoTox-Glo assay (Promega, Madison WI).
The effect on LCL-161 exposure on IAP expression was determined following exposure of Raji and Raji 4RH cells to either control treatment (DMSO 0.001%), or escalating doses of LCL-161 (1µM, 10µM, 20µM, 30µM, 40µM) for 24 hours. IAP expression levels were determined by western blot as above.

**In vivo studies**

For *in vivo* experiments 6-8 week-old severe combined immunodeficient (SCID) mice (a C.B-Igh-1 b/lcrTac-Prkdcsid /Ros MCL-mouse model) were bred and maintained at the Department of Laboratory Animal Resource (DLAR) facility at RPCI, certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in compliance with current regulations and standards of the United States Department of Agriculture and the United States Department of Health and Human Services. All the animals were housed and maintained in laminar flow cabinets or micro isolator units and provided with sterilized food and water.

*In vivo* studies utilized a disseminated human lymphoma-bearing SCID mouse xenograft model previously described. [71, 72] SCID mice were inoculated on day zero with 10 x 10^6 Raji 4RH cells through tail vein injection. After 7 days the animals were divided into four treatment groups: (1) control, (2) LCL-161 60mg/kg P.O., (3) rituximab (10mg/kg i.v.), gemcitabine (120mg/kg i.v.) and viorelbine (8mg/kg i.v.) (RGV), or (4) LCL-161 + RGV. The experiment was performed in duplicate with 5 mice per treatment group in the first experiment and 10 mice per treatment group in the second experiment. Results were analyzed in SPSS software version 21 supplied by IBM. Kaplan-Meier analysis with survival statistics was calculated using the log-rank test, and results are given as a significance (p) value. Time to development of limb paralysis served as the survival endpoint. One animal in the LCL-161+RGV combined treatment arm suffered an air embolus during RGV injection and was removed from the experiment.

**In vitro dose response studies with BMT-062789**
All dose response studies involving lymphoma cell lines exposed to BMT-062789 were performed in 384 well plates for 48 hours. Cells were loaded at a density of $2.5 \times 10^5$ cells/ml in a total volume of 30uL. Cell viability was determined with the CellTiter-Glo luminescent ATP assay purchased from Promgea. IC$_{50}$ values were calculated with GraphPad Prisim software version 6.

**In vitro apoptosis induction studies with BMT-062789**

Apoptosis induction was measured with Annexin V / Sytox Blue staining after 48 hours of exposure to the indicated treatment and concentration. Experiments were performed in triplicate. The results presented are graphs of one experiment, which is representative of all three.

**Results**

**LCL-161 has single agent in vitro anti-lymphoma activity in B-NHL cell lines**

To investigate the anti-lymphoma potential of targeting of IAP proteins in RRCLs we obtained the small molecule IAP inhibitor LCL-161 from Novartis. LCL-161 is a chemical mimetic of the endogenous inhibitor of IAPs, the second mitochondrial activator of caspases (SMAC). [73]

A panel of B-NHL cell lines incubated with increasing doses of LCL-161 for a period of 48 hours had IC$_{50}$ values in a range from sub-micromolar (WSU-DLCL2 at 0.22μM) to greater than 50μM (Raji, Granta-519, Jeko-1). The majority of cell lines tested had IC$_{50}$ values close to 40μM, with the RRCLs Raji 2R, Raji 4RH, and RL 4RH exhibiting IC$_{50}$ values of 48.77μM, 37.95μM, 43.76μM respectively (Table 3.1). These results indicate that LCL-161 is active as a single agent.
in B-NHL cell lines representing a range of disease subtypes (Burkitt’s lymphoma, mantle cell lymphoma, and diffuse large B-cell lymphoma).

*In vitro* exposure to LCL-161 alters expression of IAPs and induces apoptosis in RRCLs

To investigate the direct effects of LCL-161 on IAP levels *in vitro* in RRCLs we exposed the rituximab resistant cell line Raji 4RH and the sensitive parent line Raji to escalating doses of LCL-161 (1μM, 5μM, 10μM, 20μM, and 40μM) for 24 hours and observed a dose-dependent decrease in the expression of several IAPs, specifically cIAP1, livin, and Bruce when compared to controls (Figure 3.2a). In addition, LCL-161 decreased expression of cIAP2, but only in the RRCL Raji 4RH. It is also worth noting that several of the IAPs are primarily affected by LCL-161 at doses in excess of 10μM, which is the approximate maximum achievable dose in human.[74] These results confirm that LCL-161 can directly antagonize multiple IAPs in B-NHL cells. The altered expression of IAPs in the RRCL Raji 4RH correlated with an induction of apoptosis as evidenced by an increase in cleavage of PARP following LCL-161 exposure. LCL-161 produced a similar pattern of response in the RL and RL 4RH cell lines (Figure 3.2b). In addition to investigating the impact of LCL-161 on IAP protein expression we examined the impact of LCL-161 on caspase activation in several
cell lines that displayed increased sensitivity to single agent LCL-161. In the Karpas-422 cell line LCL-161 exposure the activity of caspases 3 and 7 in a dose dependent manner. This increase in caspase activity coincided with a pronounced drop in cell viability indicating that LCL-161 was directly triggering apoptosis (Figure 3.3a). A similar pattern of effect was observed in the U2932 cell line when exposed to escalating doses of LCL-161 (Figure 3.3b).

**LCL-161 increases the cytotoxic effect of chemotherapeutic agents in RRCLs and primary B-NHL patient tumor cells**

Due to the role of IAPs as apoptotic regulators we hypothesized that combining LCL-161 with cytotoxic chemotherapy agents would enhance their anti-tumor activity. To investigate this, we treated RRCLs and sensitive parental cells with combinations of LCL-161 and commonly used anti-lymphoma chemotherapy drugs (gemcitabine, etoposide, and vincristine). 35uM LCL-161 synergistically increased the anti-tumor effect of 50uM gemcitabine in Raji 4RH, but had minimal effect in the Raji cell line (Figure 3.4a, 3.4b). These observations are supported by the calculated combination index (CI) values for LCL-161 with gemcitabine. CI values are a measure of synergy, with anything less than one indicating a synergistic interaction. Both Raji and Raji 4RH exhibited synergistic anti-tumor responses to LCL-161 and gemcitabine, but overall a more synergistic
response was observed in Raji 4RH with 35uM LCL-161 (CI value 0.031), but synergy was observed at all doses tested. We also investigated potential synergy between LCL-161 and etoposide (Figure 3.4c, 3.4d), as well as LCL-161 and vincristine (Figure 3.4e, 3.4f). LCL-161 with vincristine was moderately synergistic at most concentrations in Raji 4RH, and only at high concentrations in Raji. The combination of LCL-161 with etoposide was exclusively antagonistic in Raji although a high concentrations of LCL-161 and etoposide did appear to be synergistic in the Raji 4RH cell line. In addition to investigating combinations of LCL-161 with conventional chemotherapy agents, we also hypothesized that LCL-161 may have synergistic activity with proteasome inhibitors based on published work that indicates targeted IAP knockdowns work with proteasome inhibitors to enhance BAX/BAK independent cell death.[75] The combination of LCL-161 with the
proteasome inhibitor carfilzomib produced a different pattern of activity (Figure 3.5a, 3.5b).

**In vivo LCL-161 significantly prolongs survival in combination with chemotherapy**

Figure 3.6 LCL-161 combined with chemotherapy can extend survival compared to chemotherapy alone in an animal model of rituximab resistant lymphoma

To determine if the observed synergy between LCL-161 and gemcitabine could improve survival in an *in vivo* TRCL model we employed a SCID mouse Raji 4RH disseminated xenograft system. Animals were inoculated with either 1x10^6 of the rituximab sensitive Raji cell line or 10x10^6 of the rituximab resistant Raji 4RH cell line. The difference in required inoculum between Raji and Raji 4RH is due to differences in engraftment fitness between the two cell lines. Following a 7 day tumor engraftment period, animals were separated into treatment and control arms.

Animals were inoculated with 1x10^6 Raji cells or 10x10^6 Raji 4RH cells. Treatment was administered 7 days after xenograft implantation. Treatment groups were LCL-161 alone (60mg/kg p.o.), RGV (rituximab (10mg/kg i.v.), gemcitabine (120mg/kg i.v.), and vinorelbine (8mg/kg i.v.)), or a combination of LCL-161 and RGV given according to the above listed doses and routes of administration. a) SCID mice inoculated with the Raji cell line responded very well to RGV with 100% survival. LCL-161 provided no additional benefit when added to RGV. 10 animals were used per group. b) SCID animals inoculated with the Raji 4RH cell line had a much poorer responses to RGV alone, although the addition of LCL-161 to RGV increased survival durations to a statistically significant degree. The figure is the result of two pooled experiments. The Control, LCL-161, and RGV groups had 15 animals total, the LCL-161 + RGV group had 14 animals total. c) Median survival duration by treatment. ‡ = Sig. value < 0.05 compared to Control. † = Sig. value 0.006 comparing RGV alone to RGV + LCL-161 by the Log Rank test. + = censored value.
was given at 10mg/kg IV. Gemcitabine and vinorelbine were given at 120mg/kg IV and 8 mg/kg IV, respectively. LCL-161 was given orally at a dose of 60mg/kg. All control arm animals inoculated with Raji survived 19 days before dying of disease. The survival duration for all animals given LCL-161 alone was also 19 days. Animals treated with RGV or RGV+LCL-161 both had a 100% survival rate (Figure 3.6a), indicating that LCL-161 provides no survival advantage in a model of rituximab sensitive lymphoma.

Animals inoculated with the rituximab resistant cell line Raji 4RH had a median control arm survival duration of 33 days, which was essentially equivalent to the median survival of the LCL-161 alone treatment arm (32 days). Treatment with LCL-161 alone produced no significant survival benefit (p=0.053). RGV alone provided a trending, but statistically insignificant extension of median survival to 53 days (p=0.089). The median survival duration of mice receiving combination therapy of RGV+LCL-161 increased to 133 days, which was a statistically significant increase in survival (p= 0.006) compared to RGV alone (Figure 3.6b).

**LCL-161 enhances the anti-tumor effect of the proteasome inhibitor carfilzomib in ex vivo lymphoma biopsy samples**

To investigate if LCL-161 could enhance chemotherapy response in a more clinically relevant model system, tumor cells derived from biopsy samples from B-NHL patients evaluated at the Roswell Park Cancer Institute were exposed to LCL-161 with or without the addition of the proteasome inhibitor carfilzomib for 48 hours after which cell viability was determined. Patients had a median age of 61 and a male to female ratio of 10/5. Patients were diagnosed with a range of indolent and aggressive lymphoma subtypes including follicular lymphoma (FL), mantel cell lymphoma (MCL), and diffuse large B-cell lymphoma (DLBCL). LCL-161 (10µM) exhibited little
to no single agent anti-tumor effect at 48 hours, which was expected given the high IC<sub>50</sub> values observed for LCL-161 in lymphoma cell lines. However, similar to the observed findings in lymphoma cell lines, LCL-161 (10µM) produced a statistically significant increase in the anti-tumor activity of carfilzomib (5nM) in several samples taken from patients with both de novo and relapsed/refractory disease (Figure 3.7). Sample 141 in particular was taken from a patient with activated B-cell diffuse large B-cell lymphoma (ABC-DLBCL) whom had failed multiple salvage therapy regimens. Descriptive statistic of the patient group are provided in (Figure 3.8).

The XIAP inhibitor BMT-062789 augments chemotherapy activity in RRCLs

Our results with LCL-161 indicated that targeting IAPs could be done
pharmacologically in RRCL models, but our *in vitro* studies indicated that XIAP was singularly important for RRCL resistance. LCL-161 has been reported to bind to XIAP; however, we hypothesized that an IAP inhibitor with greater selectivity for XIAP would have more effect in RRCL models. To investigate this hypothesis we obtained the small molecule agent BMT-062789 from Bristol Myers Squib. BMT-062789 is bivalent SMAC mimetic, which has been designed to target both the caspase 3, and caspase 7 binding domains on XIAP. This dual targeting property makes BMT-062789 (formerly compound 17) unique among currently available SMAC mimetics.[76] BMT-062789 exhibited single agent anti-tumor effect when tested against a panel of lymphoma cell lines. (Figure 3.9) IC$_{50}$ values for BMT-062789 ranged between 0.77uM in the U2932 line to 4.22uM in the RL 4RH rituximab resistant cell line. The only cell line with and IC$_{50}$ value in excess of 5uM was the rituximab resistant cell line Raji 4RH. This was not surprising given that Raji 4RH remained viable after siRNA knockdown of XIAP, while the RL 4RH cell line became apoptotic following loss of XIAP. It is also worth noting that both the Raji and RL cell lines displayed dose dependent responses to BMT-062789, while neither of these cells were affected by siRNA knockdown of XIAP.  We believe this is due to the effect of BMT-062789 on additional IAP family proteins, specifically. 

**Figure 3.9 BMT-062789 has anti-tumor activity in a range of lymphoma cell lines**

![Graph showing anti-tumor activity of BMT-062789 across different cell lines.](image-url)
cIAP1. BMT-062789 is reported to bind XIAP somewhat more strongly with cIAP1, but it is not reported to be truly selective.[76]

**BMT-062789 can increase the anti-tumor effect of chemotherapy in RRCLs**

The siRNA knockdown of XIAP in Raji 4RH cells was enough to enhance the cytotoxic activity of chemotherapy agents. We hypothesized that the combination of BMT-062789 and chemotherapy would produce a similar augmented effect. To test this we incubated cells for 48 hours with either a 1uM or 3uM concentration of BMT-062789, with or without concurrent 20uM etoposide exposure. In the Raji cell line 3uM BMT-062789 induced substantial apoptosis, which was not increased by the addition of 20uM etoposide. (Figure 3.10a) Only a small response was observed to single agent BMT-062789 at 1uM in the Raji 4RH cell line; however, the combination of 1uM BMT-062789 with 20uM etoposide did increase the rates of apoptosis compared to 20uM etoposide alone. (Figure 3.10b) The 3uM dose of BMT-062789 proved more effective, with over 80% of the Raji cells and over 90% of the RL cells apoptotic after 48 hours. (Figure 3.10c)

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**Figure 3.10 BMT-062789 increases the anti-tumor activity of etoposide in RRCLs**

a), b) BMT-062789 at a 3uM concentration induced apoptosis in the Raji cell line, but not in the RRCL Raji 4RH, however the combination of BMT-062789 with etoposide was able to increase the rate of apoptosis in Raji 4RH. c), d) Single agent BMT-062789 increase apoptosis in the rituximab sensitive cell line RL. The response in the rituximab resistant cell line RL 4RH was weaker to single agent BMT-062789, however the combination of BMT-062789 with etoposide increase the rate of apoptosis to over 70%.
Responses to 3uM BMT-062789 were less substantial in the Raji 4RH and RL 4RH cell lines, but the addition of 20uM etoposide to 3uM BMT-062789 increased the cytotoxic effect to well over 90% in Raji 4RH and over 70% in RL 4RH. (Figure 3.10b, 3.10d) These results support the results of the earlier siRNA knockdown experiments, and establish that XIAP can be pharmacologically targeted to enhance the cytotoxic effect of chemotherapy.

**BMT-062789 displays anti-tumor effect in de novo and relapse/refractory lymphoma patient samples**

A total of 6 biopsy samples were collected from lymphoma patients undergoing treatment at Roswell Park. Two samples were diagnosed as follicular lymphomas (FL), and four were diagnosed as marginal zone lymphoma (MZL). All samples were incubated with either 0.3uM or 3uM BMT-062789 for 48 hours. Responses to 0.3uM BMT-062789 were generally poor; however one sample (182) from a patient with relapse MCL did show an anti-tumor response of approximately 60%, which was greater than the response to 3uM BMT-062789 observed in the same sample.

The anti-tumor effect of 3uM BMT-062789 was generally much stronger. All six samples tested had a statistically significant

![Figure 3.11 BMT-062789 has single agent anti-tumor effect in ex vivo patient samples](image-url)

3uM BMT-062789 displayed a statistically significant anti-tumor effect in ex vivo patient lymphoma samples after 48 hours of incubation. † indicates p<0.0001. Error bars = +/-1 standard error.
response to 3uM BMT-062789 regardless of relapse/refractory status. (Figure 3.11) Patient statistics are provided in Figure 3.12. This data parallels the dose response data for BMT-062789 in lymphoma cell lines and supports the continued development of XIAP inhibitors in hematological malignancies.

**Discussion**

To date only a small number of clinical trials involving SMAC mimetics have reported results. The studies have been conducted in patients with both solid tumors and hematological malignancies, with only limited single agent anti-tumor activity reported. [77-80] These findings suggest that SMAC mimetics may be of more use as chemotherapy adjuvants, rather than direct replacements for current induction therapies. This conclusion is supported by several preclinical studies of LCL-161 in combination with the chemotherapy agent paclitaxel in lung and hepatocellular carcinoma cell lines. [81, 82] In addition, LCL-161 has been demonstrated to increase the activity of BCL-2 protein inhibitors [83], radiation [84], and kinase inhibitors. [85] Although our findings suggest that LCL-161 can induce apoptosis at higher concentrations, the data from our animal studies support the position that LCL-161 would function better when used with traditional multi-agent chemotherapy regimens.

A phase I dose escalation study of LCL-161 in patients with solid tumors established 1800mg given daily in oral tablet dosing as reliably safe, although the authors note that doses of 2100mg and 3000mg daily were tolerated in some patients, and that a formal MTD dose was not reached during the trial. A daily dose of 1800mg orally resulted in a median peak plasma concentration of 2350ng/ml, which equates to 4.69µM, while a dose of 3000mg daily produced a median peak plasma concentration of 12.77µM.[74] The 10uM LCL-161 concentration used in most of our in...
*vitro* studies falls within the range of these two extremes, supporting the translational relevance of our results. Current clinical trials involving LCL-161 are focused on patients with leukemia, myeloma, and a range of solid tumors. Two phase II studies of LCL-161 combined with paclitaxel in solid tumors have reported promising initial results, but we will have to wait until the studies are published in full to know how well LCL-161 synergized with paclitaxel.

Most of the small molecule IAP inhibitors discovered to date, including LCL-161, target the BIR3 domain, which is a conserved protein domain present on several of the IAP family members in addition to XIAP.[66] The BIR3 domain is involved in binding cleaved caspase 9, but is thought to have little to no direct interaction with the effector caspases 3 and 7. The cleaved, active forms of caspase 3 and caspase 7 are reported to interact with a region around the BIR2 domain, suggesting that small molecules that interact specifically with the BIR3 domain will not fully relieve the anti-apoptotic block created by IAPs.[66] BMT-062789 (also referred to as compound 17) is somewhat unique among small molecule IAP inhibitors in that it’s a heterodimeric molecule designed to simultaneously interact with both the BIR2 and BIR3 domains.[76] This approach has obvious advantages, but it results in a molecule that is significantly larger and more complex than many other BIR3 only inhibitors.[41, 76] In addition, it remains to be seen if this more robust approach to BIR domain inhibition produces an equally robust side effect profile. The developers of BMT-062789 were able to demonstrate *in vivo* tolerability and anti-tumor effect in murine models, but it remains to be seen if heterodimeric IAP inhibitors will be tolerable enough for human trials.

Another important aspect of XIAP inhibitors is cross reactivity with other IAP family members. While specific inhibitors of cIAP1/2 have been developed[41], highly specific XIAP inhibitors are still not available. BMT-062789 is approximately 5 fold more selective for XIAP than cIAP1, but it is still highly likely that at the doses used in this study there was some cross-reactivity. While this would probably enhance the cytotoxic effect of BMT-062789 *in vitro*, it’s also likely to
increase the side effect profile \textit{in vivo}. Genetic knockout studies in mice clearly demonstrated that loss of XIAP alone is tolerable, but loss of XIAP along with loss of cIAP1 is embryonic lethal. Curiously, combined loss of XIAP with cIAP2 is reported as tolerable, in mice at least.[67] While the embryonic lethality of XIAP/cIAP1 loss cannot be directly verified in humans the effect of XIAP loss of function alone is well characterized. X-linked lymphoproliferative disorder is caused by XIAP loss of function mutations and results in a decrease in NKT cells.[86] This suggests that selectively targeting XIAP with small molecules is likely tolerable in humans, and the results of our \textit{in vitro} studies indicate that XIAP inhibition may be a very effect anti-lymphoma strategy.
Chapter 4 – Discussion, Conclusions, Translational Relevance, and Future Directions

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The impact of XIAP in RRCLs

Cell line models of rituximab resistant lymphoma have proved an invaluable tool for the investigation of the molecular mechanisms regulating survival and chemotherapy resistance in the context of concurrent rituximab resistance. The role of the apoptotic pathway, and alterations in the BCL-2 protein family, were already well characterized survival factors in RRCLs, but this work is the first strong evidence to support an additional role for the IAP protein family in RRCLs. Our work strongly supports a model where XIAP is the keystone for chemotherapy resistance in rituximab resistant lymphoma models. In addition, our findings indicate that XIAP is a viable target for potentially novel anti-lymphoma therapies. This represents a significant advancement in the field of resistant lymphoma biology.

Although these RRCLs most likely do not replicate the full complexity of rituximab relapse/refractory lymphoma, as observed clinically, they remain the best model in the field currently. We need to view the impact of XIAP in these cell lines with the overall limitations of RRCLs in mind. Our findings strongly support a model where XIAP is the principle regulator of apoptotic resistance in rituximab resistant lymphoma; however, the regulation of apoptosis is a very complex, and delicately balanced system. This work needs be viewed in the context of the other substantial apoptotic alterations in RRCLs, specifically the loss of the pro-apoptotic BCL-2 family proteins Bak and Bax. The specific importance of XIAP in the context Bak/Bax independent cell death has been reported previously in solid tumor models, but additional work is needed to fully characterize all the interactions between these two protein families.[75]
Another important aspect of this work is the specific importance of XIAP in RRCLs models, or rather the lack of impact from XIAP knockdown observed in rituximab sensitive cell lines (Raji and RL). Although the data presented in chapter 2 indicates that the cytotoxic effect of XIAP knockdown in Raji 4RH is caspase 3/7 dependent, this cytotoxic effect was observed when XIAP knockdown was combined with concurrent chemotherapy exposure. The data presented in figure 2.3 clearly demonstrates that exposure to chemotherapy agents can cause the levels of cIAP1 and/or cIAP2 to drop. Mouse models of combined depletion of multiple IAPs indicated a clear and pronounced synthetic lethal interaction when cIAP1 and XIAP are knocked out simultaneously, but this interaction may be more toxic in mice than in human tissues.[61] Our work with the selective XIAP inhibitor BMT-062789 lends support to a model where the increase in chemotherapy response is XIAP dependent alone, but even BMT-062789 is not fully selective and can target cIAP1/2 at higher doses. An important future direction for this project would be to examine how simultaneous...
knockdown of XIAP and cIAP1/2 could potentially synergize in RRCLs. As increasingly more specific IAP targeting small molecule compounds are being synthesized, these new compounds may prove to be an important tool to exploit synthetic lethal IAP interactions in rituximab resistant lymphoma. As part of this project we attempted to investigate the impact of XIAP knockdown on RRCL chemotherapy sensitivity \textit{in vivo}, but this experiment was hindered by unforeseen technical problems. As discussed in chapter 2 and referenced in figure 2.9, the shRNA knockdown of XIAP in Raji 4RH was stable long term, but the Raji 4RH cells themselves appeared to evolve an alternate resistance pathway after several months. This result was disappointing, and it prevented us from fully characterizing the effect XIAP knockdown on RRCL chemotherapy sensitivity \textit{in vivo}.

An important future direction of this project is to develop an alternate \textit{in vivo} model to study the effect of XIAP knockdown. We believe that an inducible knockdown, or knockout, model is the...
best approach to minimize the potential for the emergence of resistance in Raji 4RH cells. Of these two potential approaches, a CRISPR/Cas9 inducible knockout system is probably the best, but this system has several significant technical hurdles that would have to be overcome, not least of which is determining if a total knockout of XIAP is even be achievable. We are in the process of developing models of Raji and Raji 4RH that stably express Cas9, which should allow us to determine the impact of total XIAP knockdown in the near future.

The evolution of resistance to XIAP knockdown in the Raji 4RH cell line also provides us with an interesting opportunity to investigate potential resistance mechanisms to IAP targeting therapies. An important future direction for this work will be to investigate which pathways in these Raji 4RH_XIAP KD resistant cells are altered compared to normal Raji 4RH cells.

**The importance of XIAP in solid tumors**

Although this work is the first evidence of a critical role for XIAP in rituximab resistant lymphoma, a substantial amount is currently known about XIAP in the context of other tumor types. In pancreatic cancer XIAP is a well-documented negatively correlated survival factor.[87] The same is true for a range of other tumor types including bladder cancer, esophageal cancer, prostate cancer, gastric cancer, breast cancer, and glioblastoma.[47, 69, 70, 88-90] In each of these disease states XIAP expression was correlated with either disease progression, and/or a negative survival prognosis.

**The clinical impact of XIAP in rituximab resistant lymphoma**

One important consideration for the studies described in the previous section is that in each the expression level of XIAP itself was the primary independent variable investigated. Our work in RRCLs demonstrated that XIAP can play a key anti-apoptotic even when total protein levels of XIAP are unchanged between sensitive and resistant cell line models. Our findings suggested
that additional factors, including impaired SMAC release, and caspase 3/7 expression can create a situation in a cell where XIAP becomes a critical survival factor without being overexpressed. Because the current studies looked primarily at XIAP expression levels, and not at the overall balance of pro and anti-apoptotic factors that interact with XIAP, the actual importance of XIAP in cancer prognosis may be much greater than currently reported. Along with one of our collaborators, we investigated the how the expression level of XIAP mRNA correlated with lymphoma outcomes and cell of origin status. Although we observed no correlation between XIAP mRNA levels and survival, we hypothesize that the balance of XIAP with other apoptotic factors remains an important resistance mechanism in lymphoma. Additional retrospective studies designed to investigate more factors in the apoptotic response, in addition to XIAP, are an important future direction for this project that our group has discussed. Another important limitation of this work is the dependence on cell lines. Although RRCLs are the best currently available model system to rituximab resistance in lymphoma, they still suffer from the same limitations of all cell line model systems. We have attempted to isolate novel cell lines from biopsy samples taken from patients with rituximab relapse/refractory disease. Our group has had some success establishing cell lines from lymphoma patient biopsy tissue, but the low number of biopsy samples available from patients with relapse/refractory disease has limited our progress with the establishment of patient derived rituximab resistant cell line. Still, this remains an important future direction of this project, and we are in the process of developing a procedure to extract usable numbers of cells from needle core biopsies, which we hope will lead to more success in this aim going forward.

The combination of IAP inhibitors with BH3 mimetics
Previous work by our group demonstrated the value of BH3 mimetics in RRCL models. Obatoclax was only able to induce moderate cell killing, but given the almost total lack of Bax and Bak expression in RRCLs this is still a rather impressive result. We hypothesize that the combination of an IAP inhibitor with a targeted BH3 mimetic, such as obatoclax or the Bcl-2 specific BH3 mimetic venetoclax, would have at least an additive anti-tumor effect in RRCLs.

The value of combining a BH3 mimetic with an IAP inhibitor becomes more difficult to predict in tumor models and disease types with intact Bak/Bax expression. Clinical trials of venetoclax in patients with chronic lymphocytic leukemia (CLL) demonstrated that if a tumor cell is highly reliant on Bcl-2 for survival venetoclax alone is sufficient to induce robust tumor cell death. In a setting such as this an IAP inhibitor would probably add little to the overall response rate, but would bring a whole new group of side effects that would require management. Clinical responses to venetoclax in lymphoma have been less impressive so far than those observed in CLL, and it is suspected that this is due to MCL1 expression compensating for loss of Bcl-2 function. In this case the addition of an IAP inhibitor with a BH3 mimetic may be more tolerable than attempting to inhibit multiple Bcl-2 family proteins simultaneously. The combination of IAP inhibitors with BH3 mimetics offers a way to target the apoptotic regulatory network at different points in the signal cascade chain, which may help enhance the effect of both therapeutic approaches, and prevent the emergence of resistance to either approach when used as a monotherapy.

**Clinical efforts to target XIAP**

To date, the only clinical trials specifically targeting XIAP involved the XIAP antisense oligonucleotide AEG35156, which demonstrated that XIAP expression can be targeted safely. In one clinical trial relapsed/refractory acute myeloid leukemia (AML) patients had an overall complete remission rate of 47% after receiving AEG35156 in combination with idarubicin and cytarabine. The CR rate (91%) was higher in AML patients with relapse disease in the
second line setting.[95] The effectiveness of AEG35156 was also investigated in hepatocellular carcinoma (HCC) where the combination of AEG35156 with the kinase inhibitor sorafenib modestly improved overall survival.[96] Improvements in response rates were not uniformly observed in all trials involving AEG35156. Additional studies in relapsed AML and pancreatic ductal adenocarcinoma demonstrated no difference between AEG35156 treatment and control groups.[94] It is worth noting that in most cases the on-target effect of AEG35156 was measured by monitoring XIAP mRNA levels, which may not be as accurate as evaluating the level of XIAP protein expression directly in the tumor.[94]

Although the studies with AEG35156 were partially successful at XIAP in tumors, there are significant limitations to RNA antisense therapy. To date, the most clinically successful approaches to target protein:protein interactions have involved small molecule compounds. BMT-062789 is arguably the best currently synthesized selective small molecule XIAP inhibitor, and it is the only one we are aware of designed to target both of the crucial caspase binding domains on XIAP, but here have been additional efforts to selectively target XIAP.

Scientists at Roche USA made the first substantial breakthrough on selectively inhibiting the XIAP BIR2 domain, which is responsible for binding the effector caspases 3 and 7. In 2013 they successfully crystalized the XIAP BIR2 domain, which lead to a deeper functional understanding of how XIAP binds caspases 3 and 7.[66] This discovery most likely lead to the synthesis of the first small molecule, selective, XIAP BIR2 domain inhibitor.[97] Unfortunately, no additional information is available about the preclinical activity of this compound, but attempts to develop XIAP selective inhibitors continue. In June of 2018 the synthesis of a novel small molecule inhibitor of the XIAP BIR3 domain was reported by a group at the University of California Riverside.[98] In addition to XIAP selectivity, the authors also reported that compound 34, their selective XIAP inhibitor, had anti-tumor activity in LCL-161 resistant cell lines. Although the clinical progress in developing a viable small molecule IAP inhibitor has stalled somewhat in
recent years, very exciting developments are occurring at the early discovery stage. We remain optimistic about next generation XIAP inhibitors may hold great promise as anti-tumor agents for the clinical management of rituximab relapse/refractory lymphoma.
References


