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An Investigation into the Potential use of β-Galactoside binding protein as a novel anti-leukaemic agent

A thesis submitted for the degree of
Doctor of Philosophy
Neil Spellacy

Trinity College Dublin
University of Dublin
March 2005
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Neil Spellacy
Acknowledgements:

At the outset, I would like to thank my supervisor Professor Mark Lawler. He provided all the support and help needed and was accessible at all times for welcome comments and observations. I would also like to thank Professor Livio Mallucci, Kings College London, who provided the βGBP protein and antibody and the initial impetus for this project. A word of thanks is also needed for Professor Shaun McCann, Dr Paul Browne and the staff of the Cryobiology laboratory, St. James Hospital and especially Dr. Nicola Gardiner for the provision of patient samples.

A word of thanks goes to the other members of the Haematology/Oncology Laboratory (initially in Sir Patrick Duns and now spread to bigger and hopefully better labs) who over the years gave the lab its unique flavour and atmosphere and provided plenty of laughs, and a daily Irish Times. There has been so many people through the lab that I would only leave someone out if I tried to name them all but they know who they are themselves, so thanks to you all.

The special word of thanks is reserved for my parents. They have supported me in everything I have done over the years and I wouldn’t have got this far without them.

And finally in case she thinks I have forgotten her, thanks to Sarah, who has been there for me through thick and thin and is a constant source of inspiration for me.
Abstract:

Chronic myelogenous leukaemia (CML) is characterised at the molecular level by a (9;22) translocation which places the abl proto-oncogene under the control of the breakpoint cluster region (bcr) gene promoter generating a fusion protein (p210) with enhanced tyrosine kinase activity. CML cells are inherently resistant to apoptosis induced by conventional chemotherapeutic agents.

The negative regulatory cytokine β-Galactoside Binding Protein (β-GBP) can induce cell cycle arrest at the late S/G2 threshold. Negation of this block by anti-βGBP antibodies allows normal cells to resume growth but neoplastic cells undergo apoptosis. Previous analysis on the activity of βGBP indicated that various cancer cell types including lymphoma cell lines and mammary cell cancer cell lines, all undergo significant levels of apoptosis following treatment with β-GBP at nanomolar concentrations, suggesting that βGBP may offer a novel and selective means of controlling neoplastic growth.

Initially the effect of βGBP on CML cell lines (K562, BV173, LAMA84, KYO-1) was assessed and levels of apoptosis of 30-50% was demonstrated in all the cell lines tested using TUNEL assays and Annexin V staining. Dual staining with Propidium iodide, which can be used to measure cell cycle distribution, showed that at 24 hours, cells exposed to βGBP had been arrested in S phase. At 48 hours, an increase in the levels of sub-G1 cells was also detected, further supporting a role for βGBP in the induction of apoptosis in CML cells at 48 hours. Thus βGBP blocks the cell cycle in CML prior to activating cell death. These results prompted analysis of the effect of βGBP on primary hemopoietic progenitor cells (HPCs) from bone marrow donors and
CML patients using *in vitro* colony forming assays. Treatment of HPCs from normal BM donors (n=22) with 400ng/ml βGBP did not significantly reduce CFU-GM number (median inhibition 0-17%, p < 0.01 at 48 hours). By contrast treatment of HPCs from CML patients at diagnosis (n=13) resulted in a significant decrease in colony number (median inhibition 49-63%, p<0.01 at 48 hours). Thus βGBP has a significant effect on the proliferative ability of leukemic progenitors while sparing normal hemopoietic progenitor cells.

Treatment of CML cells with βGBP also resulted in a disappearance of the p210^{BCR-ABL} protein after 40-44 hours incubation and this down-regulation preceded the appearance of significant numbers of apoptotic cells. Quantitation of bcr-abl mRNA levels by real time PCR using TaqMan technology indicated that mRNA levels are unchanged following treatment with βGBP indicating that p210 down regulation occurs at the post transcriptional level. The down-regulation by βGBP was found to be caspase-3 and proteasome independent. Thus two critical events, the prior introduction of a cell cycle block, allied to the subsequent down regulation of p210^{BCR-ABL} may be required for apoptosis induction by βGBP in CML cells. At a mechanistic level, treatment with βGBP resulted in a loss in the active form of ERK1 (p42) in MAPK (mitogen associated protein kinase) cascade with no alteration in the activity of either p38 or SAPK/JNK (stress associated protein kinase/Jun N-terminal kinase) proteins. A decrease in the activity of the translationally associated proteins, S6 ribosomal protein was also demonstrated. This protein is normally associated with the translation of proteins with 5' pyrimidine tracts of which p210^{BCR-ABL} is a member. These results suggest that treatment with βGBP results in a downregulation of the anti-apoptotic protein p210^{BCR-ABL} through inhibition of ERK activity and
subsequent loss of S6 ribosomal activity resulting in the inhibition of the translational of bcr-abl mRNA into protein.

These results highlight the activity of a natural cytokine against leukemic HPCs and its ability to downregulate p210^{BCR-ABL}. It may offer a new method to selectively induce apoptosis in CML cells while sparing normal hemopoietic progenitors. However it also suggests that translational proteins represent a potential target for inhibitors of the translational process and inhibitors of these proteins can downregulate anti-apoptotic proteins bringing the induction of apoptosis in otherwise resistant cells. It also suggests that inhibition of the translational process may represent a novel target for overcoming apoptosis resistance in chemotherapy resistant leukaemia cells.
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Introduction
1.1 Introduction to Haematopoiesis:
In the circulatory system, there are multiple cell types, each with separate functions and properties. One of the features of these actively circulating cells is that they are terminally differentiated, so are distinguishable and exclusive from their precursors. They also have variable life spans ranging from a few hours for granulocytes to several years for lymphocytes. Thus there is a huge turnover in cell number but the actual cell total never changes except when for example during infection or any other immune challenge. In this case, the body responds by amplifying the subset of cells needed for the response very quickly, for example plasma cells, which are needed to produce antibodies. The ability of the haematopoietic system to respond to challenges and also to maintain a continuous poll of circulating cells can be explained by the presence of more primitive precursor cells which have the ability to generate all types of mature blood cells including both myeloid and lymphoid cells. This development from precursor cells is the first step in a complex process of amplification, maturation and differentiation as outlined in Figure 1.1.
Figure 1.1: Hierarchy of Immune Cell Development

Each stage of development or maturation is controlled by the presence of specific cytokines and each generation of cells can be distinguished from the previous generation through specific maturation markers. Specific examples of important cytokines and interleukins are illustrated.
The regenerative capacity of the haematopoietic system and the ability of these stem cell to produce large numbers of all the different cell types required has been extensively studied, initially using murine systems and subsequently in human systems. From these studies, it was established that precursor cells had the ability to proliferate and reconstitute the entire haematopoietic system of $10^{11}$ to $10^{12}$ cells and maintain this production and turnover for long periods of time. (Till and McCulloch 1961) were the first to demonstrate the regenerative capacity and stem cell origin of the haematopoietic system, in that they showed that an infusion of marrow cells from a donor animal could rescue the haematopoietic system of an animal, which had been irradiated, with a sub-lethal dose of radiation. Most research in haematopoiesis now is focused on the isolation of these powerful precursor stem cells but this has proved difficult and one of the reasons for this is that the actual number of the stem cells is very small (Abkowitz, Linenberger et al. 1990).

Haematopoiesis occurs at specific sites in the body mainly in the bone marrow, with less prolific sites in the blood and spleen. Most of the differentiation and maturation takes place in the marrow compartment and terminally differentiated cells are mobilised from the bone marrow to their site of action. These primitive cells and differentiating cells are kept in the bone marrow compartment and home to their sites of action by interacting with various adhesion molecules. Different families of adhesion molecules mediate cell-cell and cell extra-cellular matrix interactions and these have been shown to be of great importance in regulating haematopoiesis. Molecules such as the integrins (e.g. LFA-1), the selectins, the CD44 molecule and the intercellular adhesion molecules (ICAM) play important roles in the process of homing and egress of progenitor cells and maintain haematopoietic cells at specific sites (Thijsen, Schuurhuis et al. 1999). Premature release of progenitor cells and
immature cells into circulation plays a role in many diseases of the haematopoietic system including haematological malignancies. Many of these diseases e.g. Chronic Myeloid Leukaemia (CML) are characterised by the premature release of large numbers of immature cells, in this case of the granulocyte lineage, in circulation.

Regulation of the process of haematopoiesis through each stage from the progenitor cell to the terminally differentiated mature cell is controlled by a large repertoire of molecules. Figure 1.1 offers a very simplified picture of the differentiation and maturation process. Multiple antigenic changes occur during differentiation and maturation and these can be used to identify each stage of differentiation and also the different cell types from each other. At each step of the process, there are a number of different molecules acting on each cell lineage to enhance or inhibit the production of a specific cell type. Most of these molecules are classified as cytokines and they act on specific receptors present on a certain cell type. Through signalling cascades these cytokines can up regulate or down regulate transcription. This allows maturation of the cell through the expression of cell surface antigens, which allow the cell to respond to further stimuli and may also inhibit responses to earlier stimuli in the differentiation pathways. This process advances the maturation and differentiation of each cell type to a final terminally differentiated cell, which bears very little resemblance to its progenitor. Terminally differentiated cells have reached the point at which no further differentiation takes place but they still retain the ability to respond to cytokines, for example during signalling maintenance in neutrophils. Cytokines (as illustrated in Figure 1.1) also control the relationship between the bone marrow matrix and maturating cells. Through the stimulation or inhibition of cell surface receptor expression, the adhesion between the cell and the matrix is reduced as the cell matures. It is only when the cell is fully mature and terminally differentiated that its
association with the matrix will be reduced to the extent that the mature cell exits the bone marrow and circulates freely. Any perturbation of this process of cytokine induced maturation and adhesion to the bone marrow compartment can have significant consequences and is more often than not one of the causes or consequences of leukaemia.
1.2 Introduction to Leukaemia:

From a medical history perspective, leukaemia is a recently identified disease. It was only first noted as a distinct condition in the middle of the nineteenth century. An immunologist called Virchow in 1847, described a patient who upon autopsy had blood which was “very thick... like gruel... resembling in consistency and colour the yeast of red wine”. The blood was noted to be of a whitish colour and this was found to be due to an alteration in the proportion of “coloured cells and colourless cells”, which are now recognised as a reversal of the red cell to white cell ratio. He coined the phrase “Weisse blut” or white blood and gave this condition the name of leukaemia. Since this initial description, the study of leukaemia has advanced rapidly with the identification of the various different forms of the disease and the elucidation of the morphological, clinical and biochemical features of the disease. More recently the molecular determinants of the disease have been elucidated providing us with a more complete understanding of the development of leukaemia.

Initial treatments of leukaemia focused on the reduction in cell number but these remissions were more often than not very short as the compounds used to treat the disease were not very selective for malignant cells and also were often quite toxic. Compounds such as arsenic oxide and various alkylating agents were studied and developed. Often these compounds proved to be of no long-term benefit in terms of increased survival. However these studies led to the development of lead compounds, which do have a role to play in treating haematological malignancies e.g. busulfan, cyclophosphamide. In the middle of the 1970’s, the discovery of the cytokines provided potential new molecules for treating both leukaemia and other haematological malignancies. These cell growth and growth regulating molecules were used therapeutically to either inhibit tumour cell growth or to stimulate normal
cell growth. Compounds such as α-interferon have also shown efficacy in the treatment of certain leukaemias particularly CML (see section 1.6.2).
1.3 Classification of Leukaemia:
The initial diagnosis of leukaemia in the nineteenth century only identified it as a condition with a wide range of symptoms but there was no classification of the different types of leukaemia. It was later determined that there was two major classifications. Acute leukaemias were identified by the presence of immature proliferating cells while chronic leukaemias were identified by the presence of more mature cells. This empirical classification method is still used but the addition of more sophisticated morphological and cytochemical methods allow each specific leukaemia type to be identified accurately. Using these methods, leukaemias were classified as either lymphoid or myeloid (granulocytic) in origin. The morphological classification criteria FAB (French-American-British) method has been used extensively for decades to identify further subtypes of leukaemias. The combination of FAB morphological criteria with modern immunological evaluation has further enhanced the classification and sub-classification of leukaemias, which allows for a much better understanding of the disease. Further refinement of immunological and cytogenetic techniques combined with the use of molecular approaches allows a more specific classification of leukaemia to be achieved. This has advantages because each leukaemia has clinical features, which influences clinical outcome so it is important to know what disease is present and so therapy can be tailored to each specific condition.

Correct identification of leukaemic subtypes is increasingly relevant for patient diagnosis and prognosis for example in acute myelogenous leukaemia (AML). AML can be divided into distinct FAB subtypes (M1-7) using immunophenotyping and molecular marker analysis.

Leukaemia is classified as an uncontrolled expansion or proliferation of haematopoietic cells that don’t retain their capacity to differentiate normally. This
abnormal differentiation is reflected in the accumulation of mutations in genes involved in essential cellular processes. Many of these mutations can be identified by cytogenetics e.g. the Ph' chromosome in CML (see below). Molecular techniques allow the genes involved in chromosomal rearrangement events to be identified. Many of the genes that are mutated in leukaemia are involved in relevant cellular processes including gene transcription, cell signalling, and cell cycle regulation. Aberrant transcription of genes, and the subsequent uncontrolled proliferation and cycling of cells, is a feature of leukaemia. Examples of genes which are activated in leukaemia include members of signalling pathways, ligands/growth factors, apoptosis proteins (both anti and pro) and cell surface receptors. These acquired genetic abnormalities result in the accumulation of cells with the ability to function in a growth factor independent fashion and escape the controls that are relevant to normal cell growth and differentiation.
1.4 Chronic Myeloid Leukaemia (CML):

1.4.1 Clinical Features of CML:

Chronic Myeloid Leukaemia (CML) is a myeloproliferative disease, resulting from the abnormal expansion of a clone of cells. Myeloid progenitors undergo a marked expansion resulting in large numbers of myeloid lineage cells being released prematurely into the peripheral blood. There is an initial increase in the numbers of mature neutrophils followed by the appearance of immature granulocyte precursors. CML is widely recognised to have three distinct phases each with distinct characteristics and clinical outcomes (Table 1.1). This chronic phase (CP) is characterised by the presence of terminally differentiated myeloid cells. Other features of this phase of the disease are splenomegaly and thrombocytosis. After a period of 1-4 years, there is a rapid progression through an accelerated phase (AP), which if not treated will result in the blast crisis (BC) phase. Transformation from the CP to AP and then BC phase is accompanied by an accumulation of undifferentiated blasts. The BC is reminiscent of acute leukaemias and the blasts may have myeloid, lymphoid, mixed or undifferentiated phenotypes (Eaves and Barnett 1996). BC manifests itself through increased leukocytosis, eosinophilia and basophilia with the presence of more immature cells. Various additional cytogenetic and molecular changes are thought to bring about the progression from CP to BC and will be discussed in section 1.4.2.

Chronic phase CML can be contained and controlled through the use of conventional therapies such as the administering of chemotherapeutic drugs like busulfan and hydroxyurea. However upon progression through the AP to the BC phase, the disease becomes extremely refractory to treatment and life expectancy at this later stage is short. The life expectancy of CML patients is 3-6 years but this greatly depends on the disease state at presentation, the time to development of BC and treatment schedule.
The peak incidence of CML is between 40 and 60 years with a median age on presentation of 53 years (Sawyers 1999) but all age groups can be affected. It is interesting to note that males are 1.4 times more likely to acquire CML (Thijsen, Schuurhuis et al. 1999). Another area of rapidly changing treatment of CML is the diagnosis of CML. The reliance on standard haematological methods has given way to the use of sophisticated cytochemical methods such as FISH, the use of panels of CD markers and more recently the use of molecular biology techniques. These techniques combined with more traditional haematology have allowed more accurate diagnoses, which can allow a more tailored treatment schedule and monitoring and also detect more rapidly the relapses that can occur. Using molecular techniques, minimal residual disease can be monitored and relapses detected before a haematological relapse manifests itself.
Phases of Chronic Myeloid Leukaemia

Chronic Phase (CP):
- No significant symptoms
- None of the features of accelerated or blast crisis

Accelerated Phase (AP):
- White blood cell count difficult to control with conventional drugs
- Rapid doubling of white blood cell count
- \( \geq 10\% \) blasts in blood or marrow
- \( \geq 20\% \) blasts plus promyelocytes in blood or marrow
- \( \geq 20\% \) basophils plus eosinophils in blood
- Anaemia or thrombocytopenia unresponsive to chemotherapy
- Additional chromosomal changes
- Increasing splenomegaly

Blast crisis (BC):
- \( \geq 30\% \) blasts plus promyelocytes in the blood or marrow

Table 1.1: Table outlining the triphasic nature of CML (adapted from [Mc Elwaine, 2000])

1.4.2 The Philadelphia Chromosome:
CML was the first cancer to be associated with a specific chromosomal abnormality. In 1960, Nowell and colleagues (Nowell and Hungerford 1960), noted the presence of a shortened chromosome 22 (called the Philadelphia chromosome) in the marrow of CML patients. Later work revealed that the presence of this Philadelphia chromosome (Ph') occurred through a reciprocal translocation between chromosomes 9 and 22.
(Rowley 1973) (Figure 1.2) The Ph' chromosome is associated with over 90% of CML patients, 20-30% of adult ALLs and 2-10% childhood of ALLs. There are also very rare cases of the Ph' chromosome detectable in multiple myeloma, non-Hodgkin lymphomas and myelodysplastic syndromes (Thijsen, Schuurhuis et al. 1999). The translocation event, t(9;22), results in the formation of a shortened chromosome 22 and an elongated chromosome 9 (Figure 1.2). At the molecular level, the t(9;22) results in the translocation of the 3' segment of the *abl* gene on chromosome 9 to the 5' end of the *bcr* gene of chromosome 22 resulting in the formation of a chimeric gene *bcr-abl*, which is transcribed into a chimeric *bcr-abl* mRNA(Figure 1.4). This gene encodes for the fusion protein p210\(^{BCR-ABL}\), which is essential and sufficient for the cellular transformation associated with CML (Daley and Baltimore 1988; Daley, Van Etten et al. 1990). The importance of this chimeric protein in CML disease pathogenesis will be discussed in section 1.5.

In the progression from CP to BC, additional cytogenetic abnormalities can occur. Loss of the Y- chromosome, 22q-, isochromosome 17, trisomy 19, trisomy 8, inversion of chromosome 16 and inversion or duplication of the Ph' chromosome are associated with the BC phase of CML.
Figure 1.2 t(9;22) translocation event in CML

Tranlocation event between chromosomes 9 and 22 results in the transfer of part of the \( abl \) gene from chromosome 9 to the \( bcr \) gene on chromosome 22 forming the Philadelphia chromosome and leading to the expression of the fusion protein \( p210^{BCR-ABL} \).
1.4.3 Molecular organisation of the Ph' chromosome:
The translocation event between chromosomes 9 and 22 involves either a breakpoint located in exon 1a or its alternative transcript 1b in the first and second intron of the \( abl \) gene. Thus the portion of the \( abl \) gene between exons 2 and 11 is transposed into the major breakpoint cluster region (M-bcr) of the \( ber \) gene giving the fusion gene \( bcr-abl \), which results in the \( p210^\text{BCR-ABL} \) protein. This type of translocation is detectable in most patients with CML and up to one third of patients with Ph (\( p210^\text{BCR-ABL} \)) positive adult lymphoblastic leukaemia (ALL). In CML, due to alternative splicing in this major breakpoint cluster region, \( bcr-abl \) fusion transcripts with either b2a2 or b3a2 junctions are formed (Figure 1.3). In approximately 5% of these cases there is expression of both fusion products. In Ph+ childhood ALL (2 – 10% of childhood ALL cases) and in very rare CML cases, the breakpoints are further upstream in the \( ber \) gene between the alternative exons e2' and e2 (m-bcr, minor breakpoint) and results in a e1a2 mRNA which give rise to the \( p190^\text{BCR-ABL} \) chimeric protein. A third breakpoint cluster termed \( \mu-bcr \) is located downstream of exon 19 and results in the e19a2 fusion gene, which produces a \( p230^\text{BCR-ABL} \) protein. \( p230^\text{BCR-ABL} \) has been associated with chronic neutrophilic leukaemia (Pane, Frigeri et al. 1996).

While it is clear that \( p210^\text{BCR-ABL} \) contributes to the pathogenesis of CML and \( p190^\text{BCR-ABL} \) Ph+ is implicated in ALL, the reason why these non-random chromosomal changes occur is not fully understood. For example, it has been speculated that the presence of ALU repeats in the breakpoint regions may influence the recombination events required for any translocation to occur (Thijsen, Schuurhuis et al. 1999).
Figure 1.3 Alternative transcripts of the p210/p190BCR-ABL fusion protein
(a) Abl, (b) Bcr genes with illustrated breakpoints. (c) p210/p190BCR-ABL fusion genes produced from the various alternative transcripts of the bcr-ABL gene due to the different breakpoints in both abl and bcr as outlined in Section 1.4.3.
1.4.4 Abl and Bcr organisation and structure:

1.4.4.1 Abl

As already explained, the t(9;22) event results in the addition of a 3' segment of the abl gene from chromosome 9 to the 5' segment of the bcr gene of chromosome 22 forming the chimeric gene \textit{bcr-abl}. As a result of this translocation a chimeric protein p210\textsuperscript{BCR-ABL} is formed. Both normal proteins (Abl and Bcr) have specific characteristics and functions which when combined together as p210\textsuperscript{BCR-ABL} contribute to the pathogenesis of CML.

Abl proteins are non-receptor tyrosine kinases, which have important roles to play in signal transduction and cell growth regulation and also have a cell cycle regulation function (Chung and Wong 1995). During the transition from G\textsubscript{1} to S phase, the Rb protein, which is bound to Abl when unphosphorylated (acting as an activation inhibitor), is phosphorylated and dissociates from Abl, thus allowing the activation of the tyrosine kinase activity of Abl. Abl itself is phosphorylated by CDC2 kinases, thus leading to its dissociation from the DNA and promoting cell progression into S phase. Treatment of CD34+ cells with antisense oligonucleotides against \textit{abl} reduced the population of cells in S phase without differentiation or apoptosis (Thijsen, Schuurhuis et al. 1999), while phosphorylated Abl protein can alter transcription by itself phosphorylating and activating RNA polymerase (Duyster, Baskaran et al. 1995).

Abl is ubiquitously expressed in hemopoietic cells and in tissues such as the spleen and thymus and is expressed as one of two splice variants. One form has a myristolation signal, which directs the protein to the plasma membrane whilst the other lacks this signal and is present in the nucleus (Wetzler, Talpaz et al. 1995). Both proteins however have a molecular weight of 145 kD. Through mutational analysis, several different motifs, which are important in Abl functioning and its role in CML.
transformation have been identified. At the NH$_3$ domain, there is homology to part of a SH3 domain, which is involved in protein-protein interactions and in binding to proline rich stretches (for outline of the Abl domains see Table 1.2) The Abl kinase activity is tightly regulated and this appears to be through the SH3 domain as insertion or deletion in the SH3 domain leads to a constitutively active Abl kinase (Mayer and Baltimore 1994). Regulators of Abl have also been shown to bind to the SH3 domain (Shi, Alin et al. 1995). Proteins such as Abi 1 and 2 (abl inhibitor protein) activate the inhibitory function of the SH3 domain. Abl, when activated promotes the degradation of both Abi proteins via the proteasome (Dai, Quackenbush et al. 1998). The importance of the SH3 domain in negative regulation of Abl kinase activity is further reinforced by the observation of a mutation from a phenylalanine (Phe$^{401}$) to a valine in the kinase domain leading to a transformation of these cells. This Phe$^{401}$ residue is highly conserved across tyrosine kinases with SH3 domains and it has been suggested that this residue may bind to the SH3 domain regulating its activity [Jackson, 1993 #223

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>p145$^{ABL}$ (chromosome 9)</td>
<td>N terminal</td>
<td>Localisation to nucleus</td>
</tr>
<tr>
<td></td>
<td>Myristoylation site</td>
<td>Negative regulation site</td>
</tr>
<tr>
<td></td>
<td>SH3 domain</td>
<td>Binding sites for tyrosine phosphorylated proteins</td>
</tr>
<tr>
<td></td>
<td>SH2 domain</td>
<td>Tyrosine kinase domain</td>
</tr>
<tr>
<td></td>
<td>SH1 domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C terminal</td>
<td>Binding sites for nuclear proteins, actin, DNA</td>
</tr>
</tbody>
</table>
Table 1.2 Position and functions of the protein binding domains of p145\textsuperscript{ABL}, p160\textsuperscript{BCR} and p210\textsuperscript{BCR-ABL}.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>p160\textsuperscript{BCR} (chromosome 22)</td>
<td>N terminal</td>
</tr>
<tr>
<td></td>
<td>Coiled-coil motif</td>
</tr>
<tr>
<td></td>
<td>Catalytic domain</td>
</tr>
<tr>
<td></td>
<td>Central</td>
</tr>
<tr>
<td></td>
<td>GDP/GTP exchange factor. Interaction with Ras family members</td>
</tr>
<tr>
<td></td>
<td>GEF</td>
</tr>
<tr>
<td>C terminal</td>
<td>GAP</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>p210\textsuperscript{BCR-ABL}</td>
<td>BCR</td>
</tr>
<tr>
<td></td>
<td>Coiled-coil motif</td>
</tr>
<tr>
<td></td>
<td>Tyrosine 177</td>
</tr>
<tr>
<td></td>
<td>Ser-Thr kinase</td>
</tr>
<tr>
<td>ABL</td>
<td>SH1 domain</td>
</tr>
<tr>
<td></td>
<td>Actin binding domain</td>
</tr>
</tbody>
</table>

Abl also contains SH2 domains, which are involved in binding phosphorylated proteins thereby linking proteins to each other. Defects in the SH2 domains decrease phosphotyrosine binding and reduce the transforming potential of Abl (Faderl, Talpaz et al. 1999). Downstream of the SH2 domain there is a tyrosine kinase/SH1 domain, the activity of which is regulated by the SH2 and SH3 domains for Abl. The Abl
protein also contains a nuclear localization signal and at its COOH domain, a DNA binding domain and an actin binding domain (Table 1.2)

1.4.4.2 Bcr

The bcr gene on chromosome 22 codes for a protein which can vary in size from 83 to 190 kD depending on which splice variant is present. Bcr is normally located in the cytoplasm in Go cells but when cells undergo mitosis, it is located in the nucleus, suggesting an important role in cell cycle regulation (Wetzler, Talpaz et al. 1995). At the NH₃ terminal, there is a dimerization domain involved in the formation of a coiled-coiled structure. A number of other domains, which are also involved in protein binding, are located 3' to the dimerization domain (for description of Bcr domains see Table 1.2). Other important domains include a segment with a unique serine/threonine kinase activity and a region involved in calcium dependent lipid binding (CaLB). The C terminus GTPase activity for Rac, which regulates actin polymerisation, also regulates the activity of an NAPDH oxidase (Diekmann, Nobes et al. 1995). The centre of the molecule contains a region with dbl like and PH (pleckstrin homology) domains that stimulate the exchange of guanidine triphosphate (GTP) on Rho guanine nucleotide exchange factors (Rho-GEF), which may in turn activate transcription factors like NF-κB (Denhardt 1996). Bcr can also be phosphorylated on tyrosine residues, the most important of which is tyrosine 177. Tyr 177 is a site for GRB2, an adaptor protein involved in the Ras pathway activation (Ma, Lu et al. 1997).

Therefore both proteins have specific motifs which play important roles in their proteins functions, but in CML when the two genes are placed together and a fusion
protein formed, these domains play an important role in the pathogenesis and transforming ability of p210$^{\text{BCR-ABL}}$.

1.4.5 p210$^{\text{BCR-ABL}}$ organisation:
As already described, normal Abl kinase activity is tightly regulated under normal physiological conditions via its SH3 domain, illustrated by the observation that an insertion or deletion in the SH3 domain leads to a constitutive active Abl kinase (Mayer and Baltimore 1994). In the p210$^{\text{BCR-ABL}}$ fusion protein, the Bcr portion of the protein is linked to the N-terminal region of Abl and this places the $abl$ gene under the influence of the $bcr$ promoter (Cotter 1995). This is a key event as it results in the production of a protein, which has an elevated tyrosine kinase activity (Lugo, Pendergast et al. 1990) and this elevated kinase activity is pivotal in the transforming ability of p210$^{\text{BCR-ABL}}$ (Daley and Baltimore 1988; Daley, Van Etten et al. 1990). However this also leads to a de-regulation of the Abl kinase activity. The positioning of the Bcr at the N-terminus of the protein interferes with the Abl SH3 regulatory domain, allowing unregulated Abl tyrosine kinase activity. It has been speculated that the Bcr portion activates Abl by a direct folding of the Bcr protein onto the SH2 domain and blocking access of the Abl SH3 domain to regulatory proteins. Mutations in the NH$_3$ domain of Bcr block the ability of p210$^{\text{BCR-ABL}}$ to transform cells (Cotter 1995), thus providing some evidence for this hypothesis.

At the NH$_3$ terminal, there is also a dimerization domain, which allows homodimers of p210$^{\text{BCR-ABL}}$ to form (Figure 1.4). This facilitates the autophosphorylation of the serine/threonine kinase domain of Bcr, activating signalling pathways mediated by Abl (Reuther, Fu et al. 1994). A deregulated Abl tyrosine kinase activity leads to further autophosphorylation of the molecule and the tyrosine residues in the chimeric protein. This creates binding sites for other SH2 domain binding proteins increasing
the number of proteins and adaptor molecules that can bind, the importance of which will be explained in section 1.5.

**Figure 1.4 The p210\textsuperscript{BCR-ABL} fusion protein**

Illustrated are the functional domains and important binding sites of p210\textsuperscript{BCR-ABL}. The importance of each domain is outlined in 1.4.4 and 1.4.5. Downstream interactions with each domain are illustrated in Figure 1.5.

This is a central feature of p210\textsuperscript{BCR-ABL}, i.e. elevated tyrosine kinase activity opening up multiple signalling pathways through which survival signals and anti-apoptotic signals can be transduced.

Another important feature of p210\textsuperscript{BCR-ABL} is its altered localisation in the cytoplasm (Van Etten, Jackson et al. 1994). The localisation is important for protein function because deletion of the COOH terminus of normal Abl delays the induction of apoptosis in drug treated cells (Okuda, D'Andrea et al. 1998). The Bcr portion can direct the p210\textsuperscript{BCR-ABL} protein to the cytoplasm and the actin-binding domain of Abl determines the precise localisation of the chimeric protein. The N-terminal coiled coil motif of Bcr increases the tyrosine kinase activity and enables binding of actin by Abl (McWhirter, Galasso et al. 1993), localising the fusion molecule in the cytoplasm. Altered localisation of p210\textsuperscript{BCR-ABL} allows the phosphorylation of cytoplasmic substrates. Therefore the normal functions of both Bcr and Abl are either changed or enhanced in p210\textsuperscript{BCR-ABL} providing for the various distinctive features of the fusion protein and the transforming potential of p210\textsuperscript{BCR-ABL}.
1.5 Altered Signalling Properties of CML cells:

Normal cell survival, growth, mitogenesis and differentiation are dependent on the receipt of extra cellular signals from the external environment in order that cellular processes can be co-ordinated between cellular populations. The inherent metabolic and signal transduction pathways of each cell are controlled by external environment and by the types of stimuli that each cell receives from neighbouring cells. These extra-cellular stimuli can take the form of either biological stimuli such as cytokines, hormones, growth factors and/or physical stimuli such as temperature changes, changes in pH or osmotic stress. In cancer cells, the normal cellular responses to these stimuli are altered in a way as to enhance and favour the survival and proliferation of the cancer cells over normal cells. Cancer cells can escape the normal controls on cellular growth and proliferation e.g. leukaemia. As already explained CML exhibits a pattern of clonal expansion and is extremely refractory to treatment by standard chemotherapies. This means that these cells must have altered signal transduction pathways and altered apoptotic pathways, which allows them to escape from the normal means of cellular regulation. The transforming ability of p210⁰⁰⁰⁰ is dependent on its kinase activity (Daley and Baltimore 1988) and on its autophosphorylation (Reuther, Fu et al. 1994), which creates multiple binding sites for adaptor proteins thereby multiplying the signalling pathways affected by p210⁰⁰⁰⁰. The proteins that are activated by p210⁰⁰⁰⁰ can be divided into at least three groups. They are, (i) adaptor proteins such as Crkl and p62DOK, (ii) proteins involved in cytoskeletal organisation and rearrangement such as paxillin and tallin, and (iii) proteins with catalytic functions such as kinase and phosphatases.

Adaptor molecules such as Crkl were found to be constitutively phosphorylated and active in CML cells (Nichols, Raines et al. 1994) and this phosphorylation by
p210\textsuperscript{BCR-ABL} allows further adaptor proteins such as SOS1 and paxillin to be recruited and be phosphorylated in turn. Constitutively phosphorylated p62\textsuperscript{DOK} is found in early CML progenitor cells (Carpino, Wisniewski et al. 1997) and active STAT family members are also found in cells containing p210\textsuperscript{BCR-ABL} (Shuai, Halpern et al. 1996). STAT activation is also associated with the activation of JAK proteins, which may act as an anti-apoptotic signal. JAK proteins have been linked to the transcriptional activation of Bcl-x\textsubscript{L} which as will be seen in section 1.5.5 is intrinsic to anti-apoptotic signalling (Horita, Andreu et al. 2000).

These adaptor molecules provide an example of the means by which p210\textsuperscript{BCR-ABL} can act to stimulate multiple proteins, which can then in turn activate further signalling cascades, thereby contributing to the disease phenotype of CML. The importance of the constitutive kinase activity of p210\textsuperscript{BCR-ABL} is illustrated by the effect of the specific kinase inhibitor STI571 (also known as Glivec). STI571 prevents the activation of the abl kinase activity of p210\textsuperscript{BCR-ABL} (see below for details) and blocks the autophosphorylation of p210\textsuperscript{BCR-ABL}, thus in addition abrogating substrate phosphorylation. In this way the transforming potential of p210\textsuperscript{BCR-ABL} is significantly reduced and significant levels of apoptosis can be induced in previously resistant CML cells (Druker, Tamura et al. 1996).

Disruption of normal cell functioning in CML cells, through the altered activity of p210\textsuperscript{BCR-ABL} and production of the leukaemia phenotype occurs through three major mechanisms (i) constitutive activation of mitogenic signalling pathways, (ii) reduced apoptosis and resistance to the induction of apoptosis, and (iii) altered adhesion.
1.5.1 Signal Transduction associated with $p210^{BCR-ABL}$:

1.5.1.1 Ras pathway:

Of the signalling pathways that are activated by $p210^{BCR-ABL}$, some are extremely important in cell survival and this is one of the reasons why CML cells are resistant to apoptosis induction. The role of the MAPK (mitogen activated protein kinase) pathways are very important in the transforming potential of $p210^{BCR-ABL}$. Autophosphorylation of Tyr177 in $p210^{BCR-ABL}$ provides a binding site for GRB2 (Pendergast, Quilliam et al. 1993), which then allows the binding of molecules such as SOS1/2 and thus stabilises the Ras protein in an active (GTP bound) state (Figure 1.4). Tyrosine phosphorylation and the constitutive activation of $p62^{DOK}$ by $p210^{BCR-ABL}$ inhibits the activity of Ras GAP proteins (Kashige, Carpino et al. 2000) thus inhibiting a key protein involved in the down regulation of Ras signalling. Ras plays a pivotal role in many survival pathways and is one of the central proteins in MAPK pathways, facilitating the amplification of extra cellular signals through the cell. Crkl and SHC can both also activate Ras and both these adaptor molecules (as seen above) are substrates for $p210^{BCR-ABL}$ (Nichols, Raines et al. 1994; Pelicci, Lanfrancone et al. 1995). Although Ras was found to be frequently mutated to a constitutive active state in many different cancers, (Blalock, Weinstein-Oppenheimer et al. 1999), activating mutations are rarely found in CML (Watzinger, Gaiger et al. 1994). It is more likely that the constitutive activation of Ras by $p210^{BCR-ABL}$ kinase activity is responsible for the role of Ras in the transforming potential of $p210^{BCR-ABL}$ in CML cells. By abolishing Ras activity via dominant negative mutants, the transforming potential of $p210^{BCR-ABL}$ was abrogated showing the importance of Ras in such a role (Sawyers, McLaughlin et al. 1995). Activation of the other related MAPK pathways, namely through JNK/SAPK (Jun N-terminal kinase/stress activated protein kinase) (Raitano, Halpern et al. 1995) and p38 have also been shown to be important for $p210^{BCR-ABL}$
activity and the enhanced survival of CML cells. In addition, activation of Raf through Ras can lead to MEK1/MEK2 and ERK mediated up regulation of gene transcription (Kang, Yoo et al. 2000). The complexity of these signalling pathways and the measure of how inter-connected they are is illustrated by the fact that Raf is speculated to phosphorylate the pro-apoptotic protein Bad, thus blocking its activity (Zha, Harada et al. 1996). Also there are reports that Ras phosphorylates the anti-apoptotic protein Bcl-2 on Ser^{70} residue promoting its activity (Ito, Deng et al. 1997).

Figure 1.5. Signalling pathways activated and associated with p210^{BCR-ABL} in CML cells.
1.5.1.2 Further signalling pathways associated with p210BCR-ABL:

Myc, which has both pro-apoptotic and anti-apoptotic roles, is important for the transformation of p210BCR-ABL containing cells. Activation of Myc is dependent on the SH2 domain of active p210BCR-ABL (Sawyers, Callahan et al. 1992) and the importance of Myc in cellular transformation is indicated by the fact that a dominant negative form of Myc can completely abrogate transformation of CML cells (Sawyers, Callahan et al. 1992). Myc signalling is associated with Ras/Raf and cell cycle proteins like E2F transcription factors and cyclin dependent kinases (cdk’s) (Zou, Rudchenko et al. 1997).

Another important protein activated by p210BCR-ABL is PI-3 kinase (phophatidylinositol 3 kinase), which is required for the proliferation of CML cells (Skorski, Kanakaraj et al. 1995). PI-3 kinase is a pivotal signalling molecule in inositol metabolism through which it can regulate gene expression. Multicomponent complexes of Crk, Crkl and Cbl, all of which are activated by p210BCR-ABL, bind to PI-3 kinase and activate it (Sattler, Salgia et al. 1997) (Figure 1.4). SHIP and SHIP-2 are both related to PI-3 kinase and are also activated by p210BCR-ABL via the binding of SHC to p210BCR-ABL. Both contribute to the activation and metabolic shifts required for CML cell growth and survival (Wisniewski, Strife et al. 1999). Downstream of PI-3 kinase is the tyrosine kinase AKT. AKT when activated by the catalytic subunit of PI-3 kinase, has as one of its key substrates the pro-apoptotic substrate Bad (del Peso, Gonzalez-Garcia et al. 1997). Expression of p210BCR-ABL up regulates the levels of Akt and PI-3 kinase (Skorski, Bellacosa et al. 1997) enhancing the activity of these proteins but also up-regulating the levels of proteins such as NF-κB. NF-κB is a target for active AKT and can transmit two signals, one an anti-apoptotic signal through the Ras pathway via PI-3 kinase and another through the induction of Myc expression.
Activated NF-κB can have multiple downstream effects through transcriptional activation of targeted genes. This illustrates clearly how p210<sup>BCR-ABL</sup> can induce a multiplicity of effects, all of which add to the ability of p210<sup>BCR-ABL</sup> positive cells to avoid normal cell cycles and controls.

A further family of proteins, which interact with and are activated by p210<sup>BCR-ABL</sup>, are the JAK/STAT proteins (signal transducers and activators of transcription). Both STAT1 and STAT5 have been reported to be constitutively phosphorylated in CML cells (Carlesso, Frank et al. 1996). Despite the fact that JAK proteins were found to be constitutively activated in v-abl transformed B-cells, activated JAK has not been found in p210<sup>BCR-ABL</sup> positive cells. It is speculated that the activation of the STAT’s bypasses JAK activation. As already stated, the Tyr 177 residue in the p210<sup>BCR-ABL</sup> molecule is the site for the binding and activation of Grb2 adaptor molecules. This site also plays a role in STAT activation as mutation of Tyr 177 blocks the interaction with Grb2 but also prevents the activation of STAT1 and STAT5 (Frank and Varticovski 1996). Phosphorylation of STAT’s induces dimerization, translocation to the nucleus and the binding to specific motifs and the regulation of transcription. The STAT’s regulate the transcription of many different proteins, but in CML cells STAT5 regulates the transcription of Bcl-x<sub>L</sub> (Horita, Andreu et al. 2000), which is an anti-apoptotic molecule and this contributes to the ability of CML to resist the induction of apoptosis. Therefore the activation of STAT5 by p210<sup>BCR-ABL</sup> mediated signalling can contribute to the growth and viability of CML cells.
1.5.2 Growth Factor Independence and Cell Cycle:
A consequence of the pleiotropic activation of proteins is to confer growth factor independence on CML cells (Daley and Baltimore 1988). These cells are no longer under the influence of external factors to induce and promote survival and growth. CML cells have also been observed to induce the secretion of growth factors thereby producing an autocrine loop independent of external stimuli (Sirard, Laneuville et al. 1994). However, a loss of growth factor dependence is associated with a deregulation of the cell cycle, which occurs due to the fact that the cells are no longer under the influence of external factors and as such, don't respond to these factors. One very important protein involved in the twin processes of apoptosis and cell cycle regulation is p53. p53 activation responds to external stimuli such as DNA damage. p53 upon sensing DNA damage is activated through negative regulation of MDM2. It then leads to up regulation of cell cycle arrest proteins such as p21 allowing repair of the damage. If the damage can't be repaired p53 goes on to activate the process of apoptosis through its association with BH3 domain proteins such as PUMA. In the context of cancer cells, p53 is the most widely mutated gene and indeed in the blast crisis phase of CML, p53 is one of the most commonly mutated genes. The CML cell cycle is stimulated continuously via the unregulated kinase activity of p210^{BCR-ABL}. The cell cycle in normal cells is a complex set of interacting proteins including cyclins, cdk (cyclin dependent kinases which activate cyclins) and associated factors which maintain the orderly progression of the cell cycle and control the processes of DNA replication and transcription and thus control cell growth and proliferation. p210^{BCR-ABL} prevents the down regulation of cdk proteins (Cortez, Reuther et al. 1997) and therefore blocks induction of cell cycle arrest. Cell cycle arrest plays an important role in the ordering of the cell cycle and allows for proof reading and damage repair. An example of the effect of p210^{BCR-ABL} on the regulation of the cell cycle...
cycle is the observation that the cdk inhibitor p27 is down regulated in p210BCR-ABL containing cells (Jonuleit, van Der Kuip et al. 2000). p27 in normal cells acts as a checkpoint control at the G1/S boundary and regulates entry into S phase through inhibition of cdk2-cyclin E complexes (Sherr and Roberts 1999). p210BCR-ABL down regulates p27 through its activation of PI-3 kinase/AKT and therefore p210BCR-ABL containing cells have no checkpoint controls and cycle without inhibition (Gesbert, Sellers et al. 2000). Cyclin D2/cdk4/6 complexes function upstream of cyclin E/cdk2 and they phosphorylate RB proteins which through E2F transcription factors regulate the progression of the cell cycle through S phase. CML cells have multiple abnormalities at these points, which contribute to the unregulated cell cycle in p210BCR-ABL positive cells. Active cyclin D levels are increased in p210BCR-ABL positive cells (Parada, Banerji et al. 2001) with down regulation of p27. This leads to an increase in active phosphorylated RB protein, which in turn induces a E2F associated transcription of S phase protein. Indeed in CML cells, there is a higher level of active E2F protein (Stewart, Litz-Jackson et al. 1995) and this is also associated with myc transcription, which as already described can play a role in p210BCR-ABL induced transformation. Compared to normal cells, the frequency of CML cells in S phase is much higher (Eaves, Cashman et al. 1986) indicating the propensity for continuous cell cycling regardless of whether growth factors are present or not. p210BCR-ABL also delays the cell cycle at the G2/M boundary and this allows the repair of any damage to the genome, which could have been induced through DNA damaging chemotherapeutic agents and so this enhances the ability of CML cells to avoid apoptosis (Bedi, Barber et al. 1995)
1.5.3 Altered Adhesion:

Normal progenitor cells rely on molecules such as integrins, selectins and intracellular adhesion molecules (ICAM's) to mediate cell-to-cell and cell to matrix interactions. This homing and retention of immature cells in the bone marrow compartment is necessary and essential for normal haematopoiesis. A characteristic of the disease phenotype of CML is the presence of immature cells in the peripheral blood indicating a defect in the adhesion to the stromal matrix of the bone marrow but also an inability of these cells to home to the bone marrow through adhesion molecules. Adhesion to the stromal layer in the bone marrow regulates cell proliferation until the maturation process is complete and so CML cells escape this regulatory mechanism. 

\[ p^{210_{BCR-ABL}} \] containing cells are deficient in some of the necessary adhesion molecules like the integrins, which are important signalling molecules in proliferation inhibition when cells are in the bone marrow matrix (Lewis, Baskaran et al. 1996) (Verfaillie, McCarthy et al. 1992). CML cells have been found to express a variant of \( \beta 1 \) integrin, which acts as an inhibitory molecule (Deininger, Goldman et al. 2000). Therefore normal signalling through these receptors is abrogated in CML cells. CML progenitors adhere significantly less to VLA4 (integrins \( \alpha 4/\beta 1 \)) and VLA5 (integrins \( \alpha 5/\beta 1 \)) binding regions of fibronectin and this may be responsible for abnormal integrin function (van der Kuip, Goetz et al. 2001). Adhesion to fibronectin through abnormal integrin binding can protect \( p^{210_{BCR-ABL}} \) expressing cells from apoptosis and this may be important in the case of chemotherapy aimed at totally eradicating \( p^{210_{BCR-ABL}} \) containing cells from the cellular matrix (van der Kuip, Goetz et al. 2001). The importance of integrins in normal cellular control and adhesion is illustrated by the reports that inhibition of \( p^{210_{BCR-ABL}} \) expression with antisense molecules restores \( \beta 1 \) integrin mediated adhesion and proliferation inhibition in
primary CML progenitors (Bhatia and Verfaillie 1998). Cytoskeletal elements such as tallin, vincullin and paxillin associate with integrin receptors and link these molecules with actin filaments which controls cell shape and motility but also links these molecules with protein kinase such as Grb2, Src and Crkl. Crkl, which is involved in the regulation of cell motility and also in integrin mediated cell adhesion through interaction with molecules such as Fak, is one of the major adaptor proteins phosphorylated by p210BCR-ABL (Sattler, Salgia et al. 1996). Vincullin, tallin and paxillin are constitutively phosphorylated by p210BCR-ABL (Salgia, Brunkhorst et al. 1995). Reports have also linked activated Crkl and Cbl with paxillin and PI-3 kinase (Sattler, Salgia et al. 1996). This links integrin stimulation with signalling through what we have already seen as pro-survival pathways. The C-terminus of p210BCR-ABL also has actin binding domains and it is speculated that this may be important in CML cell transformation (McWhirter, Galasso et al. 1993). Abnormal integrin function in CML cells is associated with various cytoskeletal proteins and signalling pathways and this coupled with the influence of p210BCR-ABL explains why CML cells are anchorage and adhesion independent, apoptosis resistant and constitutively active. A combination of each of these defects in CML leads to the situation where large numbers of progenitor and more differentiated cells are prematurely released into the bloodstream giving rise to the disease phenotype associated with CML.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>Serine/threonine kinase; activated by PI-3 kinase, can phosphorylate Bad; also known as PKB</td>
</tr>
<tr>
<td>Bad</td>
<td>Pro-apoptotic member of BH3 family; forms heterodimers with anti-apoptotic members of this family e.g. Bcl-2, when phosphorylated is released from dimers and sequestered by 14-3-3 proteins</td>
</tr>
<tr>
<td>Bap-1</td>
<td>14-3-3 protein; acts as adaptor molecule</td>
</tr>
<tr>
<td>Cbl</td>
<td>Adaptor molecule</td>
</tr>
<tr>
<td>CrKL</td>
<td>Adaptor molecule with one SH2 and two SH3 domains</td>
</tr>
<tr>
<td>p62\textsuperscript{Dek}</td>
<td>Adaptor molecule</td>
</tr>
<tr>
<td>Grb-2</td>
<td>Adaptor molecule with one SH2 and two SH3 domains</td>
</tr>
<tr>
<td>JAK</td>
<td>Can phosphorylate and activate STAT transcription factors and other signalling molecules</td>
</tr>
<tr>
<td>JNK</td>
<td>Serine/threonine kinase; phosphorylates c-Jun transcription factor; also known as SAPK</td>
</tr>
<tr>
<td>MAPK</td>
<td>Serine/threonine kinase; acts downstream of Ras; activity induced by growth factors</td>
</tr>
<tr>
<td>Myc</td>
<td>Transcription factor associated with induction of apoptosis and cell growth</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Transcription factor which regulates the expression of multiple genes</td>
</tr>
<tr>
<td>PI-3 kinase</td>
<td>Phosphorylates 3' positions of the inositol ring; activates Akt/PKB</td>
</tr>
<tr>
<td>Raf</td>
<td>Serine/threonine kinase; acts downstream of Ras and upstream of PKC; involved in growth factor signalling</td>
</tr>
<tr>
<td>Ras</td>
<td>GDP/GTP exchange protein; involved in growth factor signalling; frequently mutated in human cancer</td>
</tr>
<tr>
<td>RasGAP</td>
<td>Enhances removal of phosphate from GTP by Ras thereby inactivating Ras</td>
</tr>
<tr>
<td>SHC</td>
<td>Adaptor protein, which serves as a substrate for tyrosine kinase activity and as a docking site for SH2 containing...</td>
</tr>
<tr>
<td>Proteins</td>
<td>Role</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>SOS</strong></td>
<td>Adaptor molecule involved in transferring signal from activated receptors to downstream molecules</td>
</tr>
<tr>
<td><strong>STAT</strong></td>
<td>Transcription factors; signal transducer and activators involved in transferring signal from Jak proteins to the nucleus</td>
</tr>
</tbody>
</table>

Table 1.3 Properties and functions of proteins involved in interactions with p210\textsuperscript{BCR-ABL}
1.5.4 Resistance to Apoptosis:

Haematopoiesis consists of cell growth, division, differentiation, proliferation and ultimately cell death. As seen previously, each of these steps is controlled and regulated by the concentration and composition of various cytokines. However each cell type has a specific life span and the total number of each cell type is maintained at a steady level. The maintenance of this constant cell number is a result of balance between cell growth and division and cell death.

Cell death can be of two types, apoptosis and necrosis. Necrosis can be instigated by non-physiological conditions surrounding the cell, resulting in the cell bursting open releasing its contents into the surrounding environment. However this can result in localised inflammation and damage to surrounding tissues. Apoptosis in comparison is a more highly controlled and regulated process. Upon induction of apoptosis, there is no release of cell contents and therefore surrounding cells are unaffected.

Apoptosis, first described by (Kerr, Wyllie et al. 1972), was found to be a genetically controlled cell death program that was activated in the cell under various conditions e.g. oncogene deregulation, cell cycle deregulation, osmotic and drug induced stress and cell damage. It was determined to be necessary for the elimination of for example self-reactive thymocytes, virally infected cells and more importantly neoplastic cells. Immune privilege in sites such as the eye and thymus is maintained by apoptosis and is also the means by which the immune system only recognises foreign antigens and not self-reactive antigens (Thompson 1995).

Despite the fact that there are many different means by which apoptosis can be induced, the morphological and biochemical changes involved in apoptosis are similar in each case suggesting a common set of molecules involved. The morphological changes involved in apoptosis are generally well described. Upon induction of apoptosis, there is a loss of cell volume, the opposite of what happens in necrosis in
which cell volume increases. Chromatin condenses and the nucleus breaks up into membrane bound vesicles and this is associated with DNA and protein cleavage, increased Ca^{2+} levels, loss of mitochondrial transmembrane potential (Δψm) and the release of cytochrome c from the mitochondria. All of the cellular contents are packaged into membrane bound vesicles, which are then engulfed by infiltrating lymphocytes. The result is the orderly destruction of a cell, without affecting any other cell, or causing damage to surrounding tissues.

Various different families of proteins such as the Bcl-2 protein and the caspases are intrinsic to the apoptosis process and activation of these proteins is necessary for the induction of apoptosis. The caspase family of proteins play a pivotal role in the execution of the apoptotic process constituting the effector arm of the apoptosis pathway. They are cysteine proteases, which are synthesised as inactive precursors (pro-caspases). These proteases are activated upon receipt of specific signals e.g. from Fas receptor after binding of the Fas ligand. Some caspase prodomains contain a death effector domain (DED) through which they can bind to another DED domain on adaptor proteins such as FADD/MORT1. This target the caspase to ligand activated receptors at the cell surface where caspase activation occurs in the death inducing signalling complex (DISC). The binding of ligands such as TNF-α, Fas and TRAIL to their receptors triggers DISC formation (Ashkenazi and Dixit 1998). There are multiple caspase family members of which 13 have been identified so far. By binding to the DISC complex, the caspase cascade members are assembled and activated in turn (Figure 1.5)
**Figure 1.6 Proteins and pathways involved in the process of apoptosis induction**

Molecular interactions involved in the propagation of apoptosis from cell surface receptors to cytoplasmic effectors. The pathway illustrates only some of the major proteins involved in apoptosis induction.

Upstream caspases are activated first and these in turn activate downstream members, which can then cleave their target protein. These downstream caspases have many different targets and each member of the caspase family has a different role to play. They can be divided into inducer e.g. caspase 8 and effector caspases e.g. caspase 7, 10. Protein cleavage by caspases leads to the morphological changes associated with apoptosis i.e. disassembly of the nucleus. Caspases also cleave and inactivate
apoptotic inhibitory proteins such as Bcl-2 and Bcl-xL and kinases involved in anti-apoptotic signalling pathways such as Raf-1 and AKT (Widmann, Gibson et al. 1998). Recent evidence has also pointed to another cellular organelle being involved in the process of apoptosis and that is the endoplasmic reticulum. Protein accumulation in the E.R and also in the proteasome leads to the activation of HSP (heat shock proteins) and subsequent activation of BH3 domain only proteins such as Bim, PUMA which activate pro-apoptotic factors such as Bax, Bak and apoptosis proceeds through mitochondrial associated pathways of cell death.

Another important family of apoptosis related proteins are the BH3 domain containing proteins, all of which contain this highly conserved domain (Adams and Cory 1998) (Kelekar and Thompson 1998). The various members of this family are divided into either pro-apoptotic, anti-apoptotic or apoptosis regulatory proteins. The pro-apoptotic proteins are proteins such as Bax or Bak while anti-apoptotic proteins include members such as Bcl-xL and Bcl-2. Proteins such as Bad and Bik are regulators of the apoptosis pathway. These proteins have the ability to form homo and hetero-dimers and the ratio of pro/anti apoptotic members to one another determines whether apoptosis is induced or not e.g. Bcl-2/Bax heterodimers precludes apoptosis while Bax homodimers can induce apoptosis. An example of this is the phosphorylation of Bad by IL-3 mediated kinases. This allows the sequestration of Bad by 14-3-3 proteins and this phosho-Bad can no longer bind Bcl-2 and Bcl-xL and these anti-apoptotic proteins e.g. Bcl-2 are released to prevent apoptosis (Blalock, Weinstein-Oppenheimer et al. 1999). Pro-anti-apoptotic BH3 proteins are speculated to induce apoptosis through the formation of pores in the mitochondrial membranes allowing the release of cytochrome c (Green and Reed 1998). Bcl-2 and Bcl-xL inhibit
the release of cytochrome c, while Bax stimulates the release of cytochrome c (Schlesinger, Gross et al. 1997).

1.5.4.1 CML and apoptosis:
One of the clinical features of CML is the initial accumulation of mature myeloid cells. By studying CML cell lines, resistance to apoptosis was observed after growth factor withdrawal in growth factor dependent cells expressing $p210^{BCR-ABL}$ (Daley and Baltimore 1988). Resistance to apoptosis due to DNA damage by chemotherapeutic agents was also observed in $p210^{BCR-ABL}$ positive cell lines (Bedi, Barber et al. 1995). As previously explained the chimeric protein $p210^{BCR-ABL}$ has features of both Bcr and Abl kinases but in an unregulated state allowing the stimulation of multiple signalling pathways.

Enhanced survival signalling in $p210^{BCR-ABL}$ expressing cells are extremely resistant to apoptosis (Bedi, Zehnbauer et al. 1994) and this resistance was shown to be primarily due to the presence of the $p210^{BCR-ABL}$ (McGahon, Bissonnette et al. 1994). Cells expressing $p210^{BCR-ABL}$ have the ability to resist apoptosis induced by growth factor deprivation, Fas ligand addition, irradiation or cytotoxic insult (Bedi, Barber et al. 1995).

The expression of $p210^{BCR-ABL}$ blocks the activation of caspase-3, which as an initiator of apoptosis is necessary for activation of the caspase cascade and subsequent apoptosis (Dubrez, Eymin et al. 1998). Mitochondrial release of cytochrome c, which is necessary for caspase-3 activation, is blocked in $p210^{BCR-ABL}$ positive cells (Amarante-Mendes, Naekyung Kim et al. 1998). This blocking of cytochrome c release is speculated to be due to the up-regulation of anti-apoptotic genes such as
Bcl-2, which can be a result of the enhanced activity of Ras and/or PI-3 kinase (Skorski, Bellacosa et al. 1997) (Sanchez-Garcia and Martin-Zanca 1997). Both proteins can phosphorylate Bad and allow its sequestration by 14-3-3 proteins. Bcl-xL expression, which is another member of the Bcl-2 family of pro/anti-apoptotic proteins, is increased in p210BCR-ABL containing cells (Di Bacco, Keeshan et al. 2000). Bcl-xL is also transcriptionally activated by STAT proteins (Horita, Andreu et al. 2000) and as seen previously, STAT family members can bind to autophosphorylated p210BCR-ABL, through adaptor proteins bound and phosphorylated by active p210BCR-ABL (Figure 1.5).

Normal cell signalling through PI-3 kinase and subsequent phosphorylation and the activation of AKT leads to the dimerization of AKT and this active AKT, through its serine threonine kinase activity, phosphorylate Bad proteins (del Peso, Gonzalez-Garcia et al. 1997) (Figure 1.7). In this way Bad proteins bind 14-3-3 proteins and this frees Bcl-2 from Bad association. Bcl-2 is now free to associate with the pro-apoptotic proteins like Bax and Bak thus preventing the induction of apoptosis (Gross, Jockel et al. 1998).

Therefore it is easy to envisage a situation where constitutive activation of PI-3 kinase and AKT by p210BCR-ABL can lead to a complete abrogation of apoptosis induction. Therefore p210BCR-ABL as well as providing a continuous proliferative stimulus can also inhibit the induction of apoptosis.
The expression of p210\textsuperscript{BCR-ABL} affects multiple proteins many of which are associated with pro-survival pathways and the inhibition of apoptosis. The details of these interactions are outlined in Section 1.5.5.1.

A further mechanism by which p210\textsuperscript{BCR-ABL} inhibits apoptosis may be through the activity of NF-κB which is up-regulated by p210\textsuperscript{BCR-ABL} (Amarante-Mendes, Naekyung Kim et al. 1998). NF-κB activates and up-regulates the levels of IAP (inhibitor of apoptosis proteins) proteins which inhibit the activity of caspases -3, -7, -9 (Deveraux, Roy et al. 1998).
Signalling pathways associated with p210$^{BCR-ABL}$ involve multiple signals through both proliferative pathways and anti-apoptotic pathways and both types of signalling are inter-related. It is this ability of p210$^{BCR-ABL}$ containing cells to stimulate multiple proteins that promotes both disease development and drug resistance.
1.6 Treatment of CML:
1.6.1 Chemotherapy:
As previously described conventional chemotherapy is not very successful in CML. Complete and sustained remissions can be achieved in other leukaemia’s (ALL, AML) using intensive chemotherapy but prolonged survival rates are difficult to obtain in CML using drugs such as busulfan or hydroxyurea (Hehlmann, Heimpel et al. 1993). A reduction in leukocyte numbers can be achieved but the onset of accelerated phase and blast crisis cannot be prevented in such a manner. Despite the fact that the tumour burden is significantly reduced by such therapies, Ph’ positive cells can still be detected in the blood and marrow and relapses are a common occurrence. Also these drugs cannot prevent the transformation from chronic phase to blast crisis. Side effects are another concern and whilst hydroxyurea has few side effects, busulfan can cause aplasia and cell fibrosis. The stem cell nature of the disease and the attributes already described e.g. p210^{BCR-ABL}, means that CML progenitor cells, (which are not actively dividing) remain unaffected by drug treatment, and so can contribute to the induction of a clinical relapse. CML cells also express Mdr genes, which act as a pump to remove most chemotherapeutic agents from the cell and this type of resistance is especially important for blast crisis progression (Marks, Su et al. 1996). The expression of p210^{BCR-ABL} also abrogates apoptosis and prevents the induction and expression of pro-apoptotic proteins, so the damage that conventional therapies can have on CML cells is quite limited. As previously mentioned, one of the consequences of p210^{BCR-ABL} expression is that due to p210^{BCR-ABL} mediated cell cycle arrest any damage done to the genome of the cell by chemotherapy can be repaired via normal cell repair mechanisms (Bedi, Barber et al. 1995). Thus chemotherapy in essence acts to control the disease, reducing the clonal population and delaying the transition towards the blast crisis phenotype.
1.6.2 Interferon:

Interferon has been used successfully to treat a subset of patients with CML. Interferons are glycoproteins produced by eukaryotes in response to antigenic stimuli and have a myriad of effects from immuno-regulation to antiviral properties (for review of the use of interferon in CML see (Druker, Sawyers et al. 2001)). Of the members of the interferon family of proteins, interferon α is the most widely studied and in clinical use. Interferon α can significantly decrease the proliferation of immune cells and particularly leukemic cells (Faderl, Talpaz et al. 1999). It is therefore an extremely effective cytoreductive agent used in chronic phase CML. Between 70-80% of patients achieve a haematological remission and up to 20% achieve a cytogenetic remission (Talpaz, Kantarjian et al. 1991). Interferon α is often administered in conjunction with other chemotherapies to maximise its ability to induce its haematological and cytogenetic remissions (Cortes, Kantarjian et al. 1998). However patients can be interferon α intolerant or develop resistance to interferon α (Talpaz, O'Brien et al. 2001). These patients may then proceed either to autografting or allogeneic SCT (stem cell transplant). Interferon-α is reported to restore adhesion to CML cells. Reports suggest that interferon-α may in fact increase the adhesive potential of CML cells and thus reinstall the regulatory properties of stromal cells through the up regulation of the macrophage inflammatory protein-1α (MIP-1α), which up regulates and normalises β-1 integrin signalling (Bhatia, Wayner et al. 1994). As indicated previously abnormal β-1 integrin expression plays a role in the pathogenesis of CML and interferon-α treatment can also directly restore normal β-1 integrin expression (Bhatia, McCarthy et al. 1996) and in this way restore normal adhesion related signalling. However interferon-α treatment like other chemotherapies cannot prevent the transformation to blast crisis.
1.6.3 Bone Marrow Transplantation (BMT):

Experimental bone marrow transplantation was first tested in animal models of leukaemia in the 1950s. The discovery of the HLA system in 1969 by Snell and Gorer, lead to the development of transplant approaches for leukaemia in humans. In CML, allogeneic stem cell transplantation, using either bone marrow or peripheral blood stem cells (PBSCs) can induce haematological, cytogenetic and molecular remission in CML patients and this is the treatment of choice for this disease.

Allogeneic SCT (allo SCT) has been reported to have a survival rate of 50-60% up to 10 years after transplantation (Sawyers 1999). This rate can be influenced by e.g. patient age, donor type, and stage of disease at diagnosis. If the patient is over 40 years old the success is significantly lower than if the patient is under 40 years old. Patients in the chronic phase of the disease have a better survival rate than those transplanted in accelerated phase or blast crisis. Also the level of HLA matching between donor and recipient can determine the success of a cellular graft. However only 15 – 30% of CML patients are eligible for BMT. Matched unrelated donors (MUD) have increased the numbers of people eligible for SCT; however, there is still a subset of patients for whom SCT is not an option.

The success of SCT depends on the balance between GVHD (graft versus host disease) and GVL (graft versus leukaemia). The immune system plays an important role in determining whether a graft is successful at inducing remission or not. GVHD (graft versus host disease) is characterised by the activation of donor lymphocytes by recipient antigens and this can cause these donor cells to destroy recipient cells and tissue, which can have significant morbidity and mortality. GVHD can be reduced by either having very closely matched HLA donors i.e. most of the cellular antigens are identical between donor and recipient or by giving immunosuppressive drugs e.g. cyclosporine during the transplant procedure. Depletion of T-cells from the graft to
reduce GVHD is associated with higher relapse rates (Marmont, Horowitz et al. 1991). This is due to the GVL effect. T-cells in the donor graft recognise CML specific oligopeptides presented by MHC complexes and destroy the CML cells. It has been suggested that CD4$^+$ cells mediate GVL and CD8$^+$ cells mediate GVHD and CD8$^+$ depleted grafts have induced complete remission (Alyea, Soiffer et al. 1998). This GVL effect is exploited in patients who relapse after allo-SCT for CML. Donor lymphocyte infusions (DLI), using T cells isolated from the original allo-SCT donor is successful in treating >70% of patients who have relapsed after the original transplant. DLI at the time of molecular relapse is more effective than at the time of haematological relapse and chronic phase patients respond better that accelerated or blast crisis patients. While this approach can still be compromised by the occurrence of GVHD, it remains an important and successful approach to rescue relapsed patients. Selective depletion of specific T-cell subsets prior to DLI may help reduce occurrence of GVHD in this context. Another method that is being evaluated to control GVHD is to transduce donor lymphocytes with the Herpes simplex thymidine kinase gene and when GVHD becomes problematic, the donor lymphocytes can be killed off by ganciclovir administration, which is selective for cells containing the thymidine kinase gene (Jahagirdar, Miller et al. 2001). While this approach has been used successfully in a small number of studies, it remains to be critically examined.

Autografting or autologous transplant does not involve a transplant donor; cells are harvested from the patient who is then subjected to chemotherapy to reduce leukaemic burden. Following chemotherapy, the collected cells are re-infused into the patient. Use of growth factors may allow collection of Ph$^-$ negative cells, thus increasing the potential for inducing remission using this method. The Ph$^-$ negative precursor cells are mobilised using cytokines such as GM-CSF. Although a Ph$^-$ negative cell
population can be collected and re-infused into the patient, the potential for small numbers of leukaemic cells (Minimal residual disease, MRD) to be present in the autologous infusion means that leukaemia relapse can occur. Use of purging techniques such as mafosfamide or 5-flourouracil, treatment with different cytokines, cryopreservation, antisense killing of p210$^{BCR-ABL}$ positive cells, may help to reduce the risk of relapse due to MRD in the infused marrow but many of these treatments are at the experimental stage and require further study. As shown by the logarithm below, this can often be complicated and the relative low percentage of patients who benefit from a transplant means that other approaches must be developed and tested. These are some of the difficulties of many of the current therapies for CML. The identification of patients who are eligible for transplant is extremely important as it means that therapy can be tailored to those who will benefit the most from it and also by using the most up to date molecular biological techniques, the exact molecular changes and gene mutations can be identified and this would allow treatment schedules to be tailored for each patient. It is this combination of therapies combining traditional with more advanced and directed therapies (as will be discussed below) which offers the best future for the treatment of CML.
Figure 1.8 Treatment schedule for CML patients

Criteria used to assess CML patients for treatment using available methods. Adapted from (Sawyers 1999).
1.6.4 Novel Therapies:
1.6.4.1 STI571:

One aspect of this search for new therapies is the use of site directed therapies; drugs designed to specifically target certain sites in a protein or certain sequences in the genome. These approaches aim to significantly affect the activity of the target protein e.g. by physically blocking an active site preventing activation of the protein.

In CML, the tyrosine kinase activity of p210\textsubscript{BCR-ABL} represents an ideal molecular target, as p210\textsubscript{BCR-ABL} is only present in CML cells (Bedi, Barber et al. 1995). As the phenotype associated with CML is directly dependent on the tyrosine kinase activity of p210\textsubscript{BCR-ABL}, any means to reduce or abolish this tyrosine kinase activity will result in the growth arrest and apoptosis of leukemic cells.

Compounds such as the tyrphostins (Anafi, Gazit et al. 1993) and herbimycin A (Okabe, Uehara et al. 1992) were developed to block tyrosine kinase activity but these compounds also inhibited the kinase activity of other tyrosine kinases in the cell as they lacked the specificity needed for a direct targeting of p210\textsubscript{BCR-ABL}. Druker et al (Druker, Tamura et al. 1996) identified a derivative of a 2-phenylaminopyrimidine called CGP-57148B, which was highly specific for the kinase activity of Abl, kit and PDGF. Its most promising attribute was its ability to inhibit the kinase activity of p210\textsubscript{BCR-ABL}. CGI-57148B (now called STI571 or Imatinib mesylate) was found to bind to the nucleotide binding site of Abl and block the binding of ATP (Figure 1.9), thus inhibiting the autophosphorylation of Abl and subsequent substrate phosphorylation is intrinsic for the pathogenesis of p210\textsubscript{BCR-ABL} (Druker, Tamura et al. 1996). STI571 was found to inhibit the growth of Ph+ cell lines and the clonogenic potential of cells isolated from CML patients (Deininger, Goldman et al. 1997). The phosphorylation status of these cells was also affected indicating that the kinase activity of p210\textsubscript{BCR-ABL} was reduced by STI571. Ph- cells however were unaffected by
the presence of the compound (Druker, Tamura et al. 1996; Deininger, Goldman et al. 1997). At a molecular level, STI571 was found to induce a significant level of apoptosis in Ph+ cells (Fang, Kim et al. 2000) and restore the IL-3 dependency of these cells (Druker, Tamura et al. 1996). The levels of anti-apoptotic proteins such as Bcl-xL, XIAP and Bcl-xL were also downregulated without affecting the levels of proteins such as Bcl-2 or p210BCR-ABL (Fang, Kim et al. 2000).

![Diagram of STI571 mechanism](image)

**Figure 1.9 Mechanism of action of STI571**

Under normal circumstances, the binding of ATP by p210BCR-ABL leads to autophosphorylation and subsequent signal transduction. Upon binding of STI571 to the ATP binding site of p210BCR-ABL, ATP is prevented from accessing this site and no phosphorylation events can occur, thus inhibiting the activation of p210BCR-ABL and any downstream signalling is prevented.

At a clinical level STI571 has recently been licensed for use on CML patients after passing through Phase I-III of the clinical trials with exciting results and is marketed under the name Glivec™. The trial results can be summarised as follows: a complete haematological response was seen in 95% of chronic phase patients and a major
cytogenetic response was seen in 60% of cases. For patients in the accelerated phase or blast crisis, a haematological response was seen in 69% and 29% of cases respectively and a cytogenetic response in 24% and 16% of patients respectively (for complete review of clinical results and implications for STI571, (Druker, Sawyers et al. 2001)

From these results, it is clear that the success seen in the laboratories with Ph+ cell lines was successfully transferred to a clinical setting. However there are still questions as to the long term effects of continuous use of STI571 as resistance to the drug may develop and lead to a relapse and a more aggressive leukaemia developing. Resistance to the drug has already been observed in CML cell lines and occurs due a number of different mechanisms such as p210BCR-ABL over expression, multi-drug resistance gene expression reducing drug uptake or compensatory mutations in genes other than p210BCR-ABL (le Coutre, Tassi et al. 2000; Mahon, Deininger et al. 2000; Weisberg and Griffin 2000). Cases of resistance in CML patients especially in the later stages of the disease have also been noted (Weisberg and Griffin 2001). It has been found that between 50 and 90% of patients with haematological relapse following STI571 treatment, have p210BCR-ABL point mutations in at least 13 different amino acids kinase domain (Hochhaus, Kreil et al. 2002). Point mutations in this domain have been found to decrease the sensitivity of the kinase activity to STI (Corbin, Buchdunger et al. 2002) and prevent the drug from accessing its site of action. Other relapsed patients have been found to have amplification of p210BCR-ABL at the genomic or transcript level (Hochhaus, Kreil et al. 2002). However these cases are rare but patients on STI treatment will have to be monitored for a number of years to assess the drugs effectiveness.
Thus targeted therapy has yielded at least one molecule used successfully to treat CML patients, but there is interest in other kinase directed therapies for CML and other disease where a specific protein target is present. The compound SCH66336, which inhibits the prenylation reaction necessary for the correct sub cellular localization of Ras, is another compound under study. Ras activation plays an important role in anti-apoptotic signalling and the transformation process by p210^BCR-ABL and inhibition of Ras blocks this transformation potential (Sawyers, McLaughlin et al. 1995). SCH66336 has shown great promise in mouse models of CML where Ph+ mice remained leukemic free up to 12 months after treatment (Peters, Hoover et al. 2001).

It is clear therefore that direct targeting of a protein implicated in leukaemia development has the potential to overcome many of the difficulties faced by conventional therapies.

### 1.6.4.2 Additional Novel therapies:

Apart from Glivec there are several other novel approaches under study. The fact that there was a specific motif present in CML, namely the chimeric bcr-abl mRNA suggested that CML might be an ideal candidate for the use of antisense technology. Although this seems like a simple and logical means to downregulate p210^BCR-ABL, there are numerous problems to overcome. This strategy has been successful on other targets e.g. inhibition of integrin expression (Bhatia and Verfaillie 1998) but there have been problems with the down-regulation p210^BCR-ABL expression through such means. Delivery of the AS-ODN into the cell can often be difficult with various strategies used e.g. lipophilic conjugation, electroporation and streptolysin-O permeabilization (Spiller, Giles et al. 1998). Antisense therapy has had some success in *in-vitro* studies in the inhibition of p210^BCR-ABL and suppression of CML cell
growth has been achieved while preserving haematopoietic cells (Wu, Joshi et al. 1995), (Skorski, Bellacosa et al. 1997). The success in in-vivo studies has been minimal due to problems with delivery, backbone design, toxicity and cost (Clark 2000).

Ribozymes, which have site-specific activity, have also been used in the in vitro treatment of CML cell lines. These molecules are flanked with antisense molecules, which target the enzyme to a specific target site where they can cleave the target mRNA. Transfection with ribozymes has been shown to suppress expression of p210BCR-ABL mRNA and protein and inhibit the growth of CML cells (Wright, Wilson et al. 1993). However ribozymes have yet to be tested in clinical studies and ribozymes suffer from the same shortcomings as AS-ODNs, namely a low resistance to nucleases and a low specificity of cleavage of the p210BCR-ABL junctional sequence (James and Gibson 1998).

Apart from these approaches directed to a molecular target in CML, other approaches are being developed. Arsenic trioxide (As₂O₃) which has recently been licensed for use in the treatment in relapsed acute promyelocytic leukaemia, is now being studied as a potential treatment for CML. As₂O₃ directly affects the mitochondria inducing a loss in the mitochondrial membrane potential with a release of cytochrome c resulting in caspase activation and apoptosis (Perkins, Kim et al. 2000). As₂O₃ also down regulates p210BCR-ABL and induced significant levels of apoptosis in CML cells (Porosnicu, Nimmanapalli et al. 2001). The inhibition of p210BCR-ABL has been shown to be due to an inhibition of the translation mechanism, namely the inhibition of p70S6 kinase activity (Nimmanapalli and Bhalla 2002). The use of As₂O₃ alone or in conjunction with established treatments such as STI571 induces significant levels of
apoptosis in studies on CML cells showing the effectiveness of As$_2$O$_3$ as a potential treatment for CML (Porosnicu, Nimmanapalli et al. 2001).

Other molecules such as geldanamycin have also been studied as potential agents in CML. Geldanamycin and its analogues inhibit heat shock proteins (HSPs) and especially Hsp90. $p210^{BCR-ABL}$ is associated with Hsp90 and this association is necessary for the correct folding and trafficking of $p210^{BCR-ABL}$. Geldanamycin shifts binding of $p210^{BCR-ABL}$ from Hsp90 to Hsp70 and induces proteosomal degradation of $p210^{BCR-ABL}$ (Nimmanapalli, O'Bryan et al. 2001). Other proteosomal inhibitors under study, abrogate normal folding, protein trafficking and degradation and can also reduce $p210^{BCR-ABL}$ levels in CML cells and induce apoptosis (Dou, McGuire et al. 1999; Soligo, Servida et al. 2001). Arginine butyrate has been shown to down regulate $p210^{BCR-ABL}$ levels by transcriptional inhibition (Urbano, Koc et al. 1998). Down regulation of $p210^{BCR-ABL}$ and apoptosis induction in CML cells has also been demonstrated using the novel compounds in the PBOX (pyrrolo-1,5-benzoxazepines) family (Mc Gee, Campiani et al. 2001; Mc Gee, Campiani et al. 2002). However all of these compounds have only been examined as in vitro studies and all need to be further examined to assess their efficacy. Each of these studies indicates that there is potential for new approaches in this disease and some of the approaches tested may also give clues to the development of new approaches to target other malignancies.
1.7 Introduction to Galectins:
Lectins are carbohydrate-binding proteins, which are expressed extra-cellularly and play a key role in cell trafficking, signalling and inflammation (Cooper and Barondes 1999). Galectins are a large family of lectin proteins, which are important as regulators of immune cell homeostasis and inflammation (Rabinovich 1999). Members of the galectin family are defined by two properties, namely shared amino acid sequences and an affinity for β-galactoside sugars. They also share sequence similarity in a globular domain known as the carbohydrate recognition domain (CRD), which is encoded for by 3 exons in the LGALS (lectin, galactoside binding, soluble) genes, which encode for the members of this family. The CRD contains all the residues that interact with the carbohydrate ligands. There are 14 members of the galectin family discovered so far but only four of these have been well characterised and these are well conserved across species. The sites of expression differ for each of these four galectins e.g. galectin 1 in skeletal tissue, smooth muscle and the thymus, galectin 2 in hepatomas, galectin 3 is expressed in activated macrophages and galectin 4 is detected in intestinal epithelium (Barondes, Cooper et al. 1994).
Galectins are secreted from cells but not in a classical secretion pathway but it is speculated that this may be because they only respond to certain signals. Galectins lack the secretion signal peptide required for classical secretion. The galectin molecules are synthesised in the cytosol and released from the cell bypassing the E.R. and Golgi apparatus (Mehul and Hughes 1997). One reason that the galectin secretion pathway may differ from the classical pathway may be to segregate these proteins from other glycoconjugate ligands and allow the expression of these proteins in response to specific signals (Barondes, Cooper et al. 1994). Unlike other lectin family members, galectins only bind very few specific β-galactoside containing
glycoconjugates suggesting the specificity of the response of each galectin in its ligand binding.

Each of four well-described galectins has various different cellular functions. Like other lectins, galectins play a role in cell-cell and cell-matrix interactions and cell adhesion (Barondes 1984). The best studied of the galectin family are galectin 1 and 3. Both molecules have pro and anti-adhesive properties regulated through binding to ligands present on extra cellular proteins such as laminin and fibronectin (Rabinovich 1999). Galectin 3 has been implicated with roles in activation of mast cells and basophils through its affinity for IgE and IgE receptors (Frigeri, Zuberi et al. 1993) and subsequent modulation of the inflammatory response. There are also reports of galectin involvement in microbial infections and in tumour progression and metastasis.

<table>
<thead>
<tr>
<th>Galectin</th>
<th>Site of Expression</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectin-1</td>
<td>Most organs, spleen, thymus, B-cells, T-cells</td>
<td>Induce apoptosis, decrease TNF-α, IL-2, decrease activated T cells, Decrease macrophage function and inflammation</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>Macrophages, epithelial cells, fibroblasts</td>
<td>Involved in cell adhesion, inflammation, macrophage function, decreases IL-5 release</td>
</tr>
<tr>
<td>Galectin-7</td>
<td>Epidermal cells</td>
<td>Apoptosis of keratinocytes</td>
</tr>
<tr>
<td>Galectin-8</td>
<td>Liver, kidney, lung, brain</td>
<td>Mediates cell adhesion</td>
</tr>
<tr>
<td>Galectin-9</td>
<td>Thymus, T-cells, kidney</td>
<td>Apoptosis of immature thymocytes, Chemotaxis of eosinophils</td>
</tr>
<tr>
<td>Galectin-10/14</td>
<td>Eosinophils, basophils</td>
<td>Involved in inflammation</td>
</tr>
<tr>
<td>Galectin-12</td>
<td>Adipocytes</td>
<td>Apoptosis of adipocytes</td>
</tr>
</tbody>
</table>

Table 1.4 Properties and characteristics of members of Galectin superfamily
1.7.1 Galectin-1:
For the purposes of this study, the properties of most interest are those linking galectins and especially galectin 1 with immune response regulation. Galectin 1 (Gal-1) is a homodimer of two 14 kD subunits but can also function as a monomer (see below) and is evolutionally conserved and developmentally regulated. The preferred substrate of Gal-1 is lactosamine but it will also bind laminin, fibronectin and certain other cell surface membrane proteins (Perillo, Uittenbogaart et al. 1997). As mentioned previously galectin 1 is synthesised without a signal peptide and remains associated with the extra cellular matrix by binding to saccharide ligands on several different glycoproteins (Nguyen, Evans et al. 2001). The property of most interest of Gal-1 is as an immune regulator. Gal-1 can bind CD45 (a transmembrane protein tyrosine phophatase) and also a thymocyte marker (Thy1) (Symons, D et al. 2000). CD45 is necessary for efficient activation of lymphocytes through the antigen receptor and ligation of CD45 by Gal-1 leads to apoptosis (Perillo, Pace et al. 1995).

Expression of Gal-1 is present at sites of T-cell death during development and maturation and is absent on resting T-cells. Therefore only activated T-cells undergo apoptosis when exposed to Gal-1. The level of expression of the product of the LGALS gene (namely galectin 1) is elevated after 3- 4 days of CD8 T-cells activation. The presence of Gal-1 protein late in T-cell activation means that it may be acting to silence any further immune activation and induction of reactive and self-reactive T-cells (Blaser, Kaufmann et al. 1998). Galectin-1 therefore acts as a regulator of the immune response and this role is activated by the appearance of specific sequences of oligosaccharides on the cell surface, leading to Gal-1 binding and CD45 cross linking and apoptosis induction (Nguyen, Evans et al. 2001). Gal-1 can also modulate the function of the T-cell receptor (TCR) and can inhibit the production of IL-2. There is also speculation that it can act to modulate ERK signalling to alter distinct TCR
functions (Vespa, Lewis et al. 1999). It is also implicated in the activation of AP1 and down regulation of Bcl-2 leading to galectin 1 induced apoptosis (Rabinovich, Alonso et al. 2000). Gal-1 is also expressed on thymocytes and induces apoptosis in both negatively selected and non-selected cells (Perillo, Uittenbogaart et al. 1997) and is also highly expressed in areas of immune privilege such as the placenta. This suggests that Gal-1 may have a role to play in generating and maintaining immune tolerance. From this type of observation a clinical use for Gal-1 has emerged. Rheumatoid arthritis (RA) is an autoimmune disease where self reactive T-cells destroy tissues. In a laboratory model of RA, these self-antigen activated T-cells were removed by apoptosis after treatment with a recombinant Gal-1. This may therefore offer a means to down regulate self-reactive T-cells in RA and other autoimmune disorders (Rabinovich, Daly et al. 1999).

1.7.2 Identification of βGBP:
Initial work by Wells and Mallucci (1991) had identified a protein factor secreted by mouse embryo fibroblasts (MEF), which could control and inhibit cellular proliferation. Further work by this group using affinity chromatography and fractionation isolated the protein factor responsible for this inhibitory activity. They identified a protein of Mr 15,000 kD, which was identified as a β galactoside binding protein, a soluble member of the lectin super family and the monomeric form of galectin 1 (see above). βGBP’s were known to exist as dimeric lectins but work by Wells and Mallucci (Wells and Mallucci 1991) revealed that the observed proliferation inhibitory property was independent of lectin cross-linking and indeed independent of its sugar binding domains. A glycan complex was also observed to be present on a proportion of βGBP molecules (Wells and Mallucci 1992) and the presence of this glycan complex masks the saccharide binding domains and so this molecule acts
independently of its lectin characteristics. Binding of \( \beta \)GBP was determined to be through specific receptors present at \( 5 \times 10^5 \) receptors/cell and with a \( K_d \) of \( 10^{-10} \)M and retained its ability to function despite the cells being saturated with non-specific sugar binding (Wells and Mallucci 1991).

\( \beta \)GBP was found to function in a similar way to cytokines as opposed to lectins as the growth inhibition that was induced by addition of \( \beta \)GBP was due to a cell cycle block at \( G_0 \) and at the \( S/G_2 \) traverse. Removal of the \( \beta \)GBP molecule by incubation with an anti-\( \beta \)GBP monoclonal antibody allowed the cells to revert to normal cell cycling without adverse effect. Therefore a novel member of the lectin super family was observed to have cytostatic effects and function in a means similar to a cytokine (Wells and Mallucci 1991). The position of the \( \beta \)GBP gene was determined to be on chromosome 15 in the murine genome and on chromosome 22 in the human and this mapped to the position of the LGALS gene and to an region including the sis/PDGF gene (Baldini, Gress et al. 1993).

### 1.7.3 Characterisation of \( \beta \)GBP:

As mentioned earlier, the homodimeric form of \( \beta \)GBP, galectin 1 has a wide range of functions including both as a lectin and in a means similar to a cytokine. However the 15kD monomeric form \( \beta \)GBP can function independent of its lectin properties and indeed can act as a cytokine.

\( \beta \)GBP can induce cell cycle arrest at \( G_0 \) and the \( S/G_2 \) traverse. This reversible block has been observed in various different cell types from T-lymphocytes, B-cells and mammary cells (Wells, Davies et al. 1999) (Allione, Wells et al. 1998) (Novelli, Allione et al. 1999). The significance of this cell cycle block and loss of cellular
proliferation is as yet unknown but some important observations have been made and form the basis of most of the work described in this thesis.

Given that the dimeric product of the LGALS gene, galectin 1 played a role in immune regulation and modulation, it was suggested that the monomeric product of this gene would have a similar role. This was indeed the case as βGBP was found to be expressed on antigen stimulated CD4 and CD8 cells (Blaser, Kaufmann et al. 1998). βGBP was also found to down regulate the immune response and inhibit proliferation after the peak response and block further induction of naïve T-cells. But as already mentioned, βGBP induces a cell cycle block. In activated T-lymphocytes, this cell cycle block at the S/G₂ interface also triggers the production of interferon γ (IFN-γ) receptor α and β chains and this can then trigger apoptosis through binding of IFN-γ to this receptor. The induction of the production of the α and β chains begins 24 hours after exposure to βGBP and peaks at 72 hours after exposure and at this time, significant levels of apoptosis can be induced by the presence of interferon-γ (Allione, Wells et al. 1998). This is one of the means by which βGBP can down-regulate the immune response and induce a switch from proliferation to apoptosis.

An interesting observation was also made when neoplastic cells were treated with βGBP, a cell cycle block was induced but significant levels of apoptosis were evident (Novelli, Allione et al. 1999). The cell cycle of βGBP treated normal cells were also blocked but no apoptosis was induced and upon removal of βGBP, the cells returned to a normal cycling profile. Therefore there was a selective induction of apoptosis by βGBP in neoplastic cells compared to normal cells. This scenario was observed in various different cells types for example mammary cancer cells (Wells, Davies et al. 1999) and T-cell lymphomas (Novelli, Allione et al. 1999).
The actual processes involved in this cell cycle block and selective apoptosis induction are still as yet unknown and further study is required. However one clue has emerged. βGBP was found to alter the Bcl-2/Bax ratio (Novelli, Allione et al. 1999) by down regulating the anti-apoptotic protein Bcl-2. Galectin-1 (the dimeric form of βGBP) was also found to down regulate Bcl-2 and induce Jun and AP-1 dependent transcription, which favoured apoptosis (Rabinovich, Alonso et al. 2000)

The induction of a cell cycle block and the induction of significant levels of apoptosis in neoplastic cells served as a starting observation to the work described in this thesis.
**Hypothesis of Study:**

The hypothesis behind this study was derived from previous work carried out by Mallucci et al, which indicated that treating neoplastic cells with βGBP induced significant levels of apoptosis and a cell cycle block. This induction of apoptosis however didn’t occur when normal cells were treated with the same levels of βGBP. In the situation of CML, apoptotic resistant cells were present in a background of normal cells. It was therefore hypothesised that βGBP may have the ability to induce apoptosis in these CML cells and leave the normal cells unaffected and allow repopulation of the immune system. The experimentation contained in this thesis aims to address this hypothesis.

**Objectives:**

1. To examine the effect of βGBP on the proliferation and growth of CML progenitor cells versus the effect on normal BM (bone marrow) cells
2. To determine the ability of βGBP to induce apoptosis in CML cell lines and the mechanism of this apoptosis induction
3. To establish the effect of βGBP on the expression of p210^{BCR-ABL}
4. To ascertain the effect of βGBP on cellular signalling in apoptosis resistant CML cell lines and determine the means by which βGBP elicits its biological effect
Chapter 2

Materials and Methods
2.1 Cell and Tissue Culture:

2.1.1 Cell lines:
The CML cell lines used in this study were: K562, BV173, LAMA84 and KYO-1 (all gifts from Dr. Junia Melo, Department of Haematology, Hammersmith Hospital, London).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chimeric bcr-abl mRNA</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV173</td>
<td>b3a2</td>
<td>Lymphoid</td>
</tr>
<tr>
<td>K562</td>
<td>b2a2</td>
<td>Erythroid</td>
</tr>
<tr>
<td>KYO-1</td>
<td>b3a2</td>
<td>Myeloid</td>
</tr>
<tr>
<td>LAMA 84</td>
<td>b2a2</td>
<td>Myeloid</td>
</tr>
</tbody>
</table>

Table 2.1: Chronic Myeloid Leukaemia (CML) cell lines used throughout this study and the chimeric form of the p210^{BCR-ABL} present in each cell line.

Each of these CML cell lines was positive for the Philadelphia chromosome, the t(9;22)(q34;q11) translocation and expressed the p210^{BCR-ABL} protein. They differ from each other in the chimeric mRNA species formed following transcription and splicing; b3a2 and b2a2 represent the two commonest forms of bcr-abl gene (Table 2.1). The cell lines, HL60 and Jurkat E6.1, which were also used in studies in this thesis, do not contain these translocations and were used as negative control cell lines in experiments described in this thesis. HL60 cell line was purchased from ATCC (Manassas, Virginia, USA) the Jurkat E6.1 cell line was a gift from Michael Freely, (Royal College of Surgeons, Ireland). All the cell lines were routinely screened for mycoplasma infection and contamination using a commercially available fluorometric method (Roche, Sussex, United Kingdom) (see Appendix 1 for outline of procedure).
2.1.2 Cell and Tissue Culture:
All cell lines and primary mononuclear cells isolated from patient samples were routinely cultured in RPMI 1640 Glutamax (Gibco, Paisley, U.K.) supplemented with 10% Foetal Bovine Serum (Gibco) and 1% penicillin-streptomycin (Gibco). All cells were maintained at 37°C and in a 5% CO₂ controlled atmosphere.

Cells were maintained in exponential growth by sub-culturing every 48-72 hours or once the cells have reached the required density for experimentation. Cell density experiments were performed by first assessing the cell number with a haemocytometer. 100 µl of the culture was taken and added to 200µl of an ethidium bromide: acridine orange solution. A sample of this mix was visualised under a fluorescent microscope where the cell number could be quantified. The number of viable cells shows up clearly with viable cells fluorescing green and non-viable fluorescing orange under fluorescent light. Cell number can then be calculated according to the following formula:

\[
\text{Average no. of cells per grid} \times 3 \times 10^4 = \text{number of cells/ml}
\]

The required dilution can then be made either for sub-culturing or for experimentation using fresh culture media.
2.2 βGBP (β-Galactoside Binding Protein) protein and Antibody:
The βGBP recombinant protein used throughout this study was a gift from Professor Livio Mallucci, Kings College London. The recombinant protein was diluted to the stock and working concentrations using PBS. A number of working concentration were tested - 400ng/ml, 100ng/ml or 50ng/ml. Anti-βGBP antibody which was produced from BALB/c-NS-1 cells (Wells and Mallucci 1991) was also a gift from Professor Mallucci and used at working dilution of 10μl/ml (as suggested by Professor Mallucci).

2.3 Assessment of the effects of βGBP on primary cells:
2.3.1 Isolation of mononuclear cells from patient samples:
Material was collected from normal bone marrow donors and CML patients at diagnosis (with informed consent) (the ages and disease stage of each patient is outlined in Table 4.1). The bone marrow samples were diluted 1:2 with RPMI (plus 10% FBS and 5% PS). 10mls of sterile Lymphoprep (Nycomed, Oslo, Norway) was placed in a 25ml tube and diluted marrow was layered over the Lymphoprep. This was centrifuged (Thermo IEC, Basingstoke, U.K.) for 25 minutes at 1800rpm. The mononuclear cell fraction/buffy coat was removed to another tube and centrifuged again at 1400rpm for 10 minutes to pellet the cells and remove any remaining Lymphoprep. The supernatant was removed and the cell pellet was washed once in PBS and centrifuged again. The cells were reconstituted in RPMI to the required concentration, 2 x 10^6 cells/ml (or required concentration) using RPMI-Glutamax plus FBS and pen/strep (Gibco).

2.3.2 Colony Forming Unit Granulocyte Macrophage (CFU-GM) assay:
Mononuclear cells isolated according to the protocol 2.3.1 were then used to assess the effect of βGBP on (a) normal haematopoietic progenitors and (b) leukemic
progenitors. Samples were treated as follows: $2 \times 10^6$/ml cells were treated with 
$\beta$GBP (400ng/ml, $1 \times 10^{-8}$M) or control diluent (phosphate buffered saline), and kept
at 37°C for required time period after treatment. At the required time points, 200μl of
each culture was taken, equivalent to $4 \times 10^5$ cells and treated with anti-$\beta$GBP
antibody (20μl/ml) for two hours at 37°C. This solution was then added to 1.8ml of
methylcellulose assay media (see Table 2.2 for composition). Each set of cells was
then plated with 500μl per well (giving final cell concentration of $1 \times 10^5$ cells/well)
and in triplicate and grown on for 14 days at 37°C. Colonies were counted after 14
days growth and colonies were scored as CFU-GM's when they contained $\geq 30$ cells
per colony.

<table>
<thead>
<tr>
<th>Media Component</th>
<th>Volume (ml) (per sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal Bovine Serum (GibcoBrl)</td>
<td>0.55</td>
</tr>
<tr>
<td>BSA (10% in IMDM) (Boehringer Mannheim, Sussex, United Kingdom)</td>
<td>0.18</td>
</tr>
<tr>
<td>RPMI (GibcoBrl)</td>
<td>0.29</td>
</tr>
</tbody>
</table>
| StemPro methylcellulose/2.3% IMDM (Boehringer
  Mannheim)                                           | 0.58                     |
| GM-CSF (Boehringer Mannheim) at 5IU/ml                | 0.2                      |

Table 2.2: CFU-GM assay media composition
Volume of each constituent per 1ml of CFU-GM media required for the CFU-GM short-term culture
assay in 2.3.2.
2.4 Apoptosis Assays:

2.4.1 TUNEL assay (Nuclear Fragmentation):

1 x 10^6 cells were treated with either 50ng/ml βGBP (unless otherwise stated) or with control diluent in time frame experiments (varying from 24 to 96 hours) Cells from both the treated (+βGBP) and untreated (-βGBP) cultures were harvested at each of the time points and the cells were collected by centrifugation at 1100rpm for 5 minutes. The resulting cell pellet was washed once in PBS and collected again at 1100rpm for 5 minutes. 1ml of 1% formaldehyde (in PBS) was added to the cells and left on ice for 15 minutes to fix the cells. This solution was again washed once with PBS and resuspended in 1 ml of ice-cold 70% ethanol and either left for 30 minutes at 4°C before immediate use or at -20°C overnight.

After this fixing period, the cells were again collected by centrifugation at 1100rpm and prepared for the elongation reaction. The elongation reaction mix for the TUNEL assay was prepared as follows: (per reaction) 18.9μl MilliQ water, 2.5μl TdT buffer (1M Na cacodylate pH 7.0, 1mM DTT, 0.5mg/ml bovine serum albumin), 2.5μl CoCl₂ (25mM) (Roche), 1.0μl Bio-16 dUTP (50nmol/50μl) (Roche), 0.1μl TdT enzyme (25U/μl) (Roche). Each sample was washed once in PBS and resuspended in 25μl of elongation reaction mixture. Incubation of this mixture was performed at 37°C for 30 minutes.

For the staining part of the reaction, the cells were collected after centrifugation and washed and resuspended in 50μl staining buffer per sample. The staining buffer was prepared as follows: (per reaction) 27.1μl MilliQ water 12.5μl SSC (20X)- (0.3M sodium citrate, 3M NaCl pH 7.0), 10.0μl Blotto (25% solution), 0.35μl Avidin-FITC (Sigma)*, 0.05μl Triton X 100. The reactions were incubated for 30 minutes in the dark. After this time period, samples were washed once in PBS and resuspended in a
solution of PBS containing 1μl/ml propidium iodide. 0.5μg/μl DNase free RNase was added to this solution and this was incubated at room temperature in the dark for 15 – 30 minutes. Analysis of the resulting samples was carried via flow cytometry (FACsCalibur, Becton Dickinson)

*1mg in 250μl PBS, 1/10 dilution of this in MilliQ water = 160X stock

2.4.2 Annexin V assay:
As with protocol 2.3.1, 1 x 10⁶ cells were treated with 50ng/ml βGBP or control diluent for various time points (24 to 96 hours). The cells were collected at the required time points by centrifugation at 1100rpm for 5 minutes and washed once in Annexin V binding buffer (Biosource, Nivelles, Belgium). Each sample was resuspended in 100μl of binding buffer to which 3μl of Annexin-V-FITC (IQ Products, The Netherlands) had been added. This solution was incubated for 15 minutes on ice in the dark. After washing once in binding buffer, the cells were resuspended in 0.5ml of binding buffer. A solution of binding buffer containing 250ng/ml PI was prepared and 0.5ml of this solution was added to each tube (final PI concentration now 125ng/ml) just before collection of data by flow cytometry (FACsCalibur, Becton Dickinson).

2.5 Assessment of the effect of βGBP on downstream signalling:
2.5.1 Detection of active Caspase-3:
1x 10⁶ cells were treated with 50ng/ml βGBP or control diluent for times ranging from 24 to 72 hours. At the required time points, the cells were harvested by centrifugation and washed once with PBS. Samples were fixed and permeabilised using Cytofix/Cytoperm (Becton Dickinson) for 20 minutes at room temperature. The cells were pelleted at 1400 rpm for 5 minutes and washed once with Perm/Wash
buffer (Becton Dickinson) and stained with 20μl anti-active caspase-3 (Becton Dickinson) for 1 hour in the dark at room temperature. After this the cells were washed once with Perm/Wash buffer (Becton Dickinson) and analysed via flow cytometry (FACSCalibur).

2.5.2 Western Blotting:

2.5.2.1 Protein extraction:
In order to extract protein for use in western blotting experiments to assess the effects of βGBP treatment, cells were harvested at the required time points and washed once in PBS. Cells were then lysed in lysis buffer containing (1M Tris-HCl pH 7.4, 1M NaCl, 0.1 M Na pyrophosphate, 1mM NaF, 1% IGEPAL CA-630, 10% SDS, 1μg/ml leupeptin, 1M pepstatin, 1mM sodium orthovanadate, 1.5μg/ml aprotinin) for 15 minutes on ice and solution was cleared by centrifugation at 12,000g for 15 minutes at 4°C.

2.5.2.2 Protein Quantitation:
Protein concentration in extracted lysates was assessed by the Bradford assay. Known standard concentrations of BSA were set up to contain 0, 1, 2.5, 5, 7.5, 10, 15μg/100μl and 100μl of each of these solutions was added to 100μl of Bradford solution (Sigma) (diluted fresh 1:2.5) in a 96 well plate. 1μl of the protein solution was added to 99μl of water and 100μl of Bradford solution added to this in a 96 well plate. OD_{595nm} was read using a Spectra Fluor Plus (Tecan, Reading, United Kingdom) and a standard curve plotted using absorbencies from standard solutions. From this standard curve, the concentration of the protein lysates could be determined.
2.5.2.3 SDS PAGE and Protein Transfer:
In order to facilitate the detection of specific proteins by Western Blotting, the lysates prepared above were electrophoresed on an SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) system, which separates protein species based on their size. The first step of this procedure was the construction of the required acrylamide gels. The resolving gel was then prepared as follows and an 8% gel was used unless otherwise stated: (per gel) 3.22ml ddH₂O, 1.82ml 30% acrylamide, 1.82ml 1.5M Tris-HCl pH 8.8, 70µl 10% SDS, 70µl 10% ammonium persulphate, 2.8µl TEMED. This mix was poured between a set of glass plates to form the actual gel, to within 1cm of the bottom of the comb, which forms the wells into which samples are placed. This was overlayed with isopropanol and allowed to set for 20 min at room temperature. After setting, the isopropanol was rinsed off and the stacking gel prepared. This was made up of: (per gel) 1.4ml ddH₂O, 330µl 30% Acrylamide, 250µl 0.5M Tris-HCl pH 6.8, 20µl 10% SDS, 20µl 10% ammonium persulphate, 2µl TEMED. This was poured into the gel sandwich and the comb inserted and this gel sandwich was allowed to set for 15 min. Upon setting the gel sandwich was placed into its correct position in the gel rig, and the rig filled up with running buffer (0.025M Tris-HCl, 0.192M Glycine, 0.2% SDS). The comb was removed and the wells were washed out with running buffer. Samples were prepared by aliquoting out the required concentration of protein as per the Bradford assay. An appropriate amount of 2X protein dissociation buffer: 0.5M Tris-HCl pH 6.8, 10% SDS, 0.2M EDTA, glycerin, bromophenol blue, 2% β-mercaptoethanol was added and the samples were boiled at 100°C for 5 min. The gel was electrophoresed at 20mA per gel for 1.5-2 hours or until the marker dye had reached the end of the gel.
Once electrophoresis was completed, the gel plates were separated and the stacking gel removed. Filter papers and a nitro-cellulose membrane (Pharmacia Biotech, Buckinghamshire, U.K.) were pre-cut to size and soaked in transfer buffer: 14.4g/l glycine, 3g/l Tris-HCl, 1g/l SDS which was made up in 800ml H₂O and made up to 1l with methanol (pH 8.2-8.4). Three filter papers and the nitro-cellulose membrane were first placed on the transfer cassette and the gel overlaid over these. Three more filter papers were then placed over the gel and this “sandwich” was rolled to expel any bubbles. This sandwich was cut to the exact size of the gel and rolled again to remove any remaining bubbles. This "sandwich" was placed in the tank of the transfer system being used (BioRad, Hertfordshire, U.K) and the tank filled up with transfer buffer. The apparatus cover was then replaced and the gel was transferred for 90 min at 160mA.

After transfer, the membrane could be then either frozen at -20°C for later use or used immediately for Western blotting.

2.5.2.4 Immunodetection:
Membranes were blocked by incubation for 1 hour at room temperature in 5% blocking solution (5% Marvel milk in TBS containing 0.05% Tween-20) unless otherwise stated. The blots were incubated with different antibodies depending on the protein of interest. Each primary antibody was made up in a different solution and at varying dilution factors, each determined by titre. Depending on the specificity of the primary antibody, differing secondary antibodies were used. The following are the protocols for the primary antibodies routinely used:

- anti- human abl (Calbiochem, Nottingham, UK) reconstituted at a concentration of 1:200 in 5% blocking solution and anti-mouse horseradish peroxidase (HRP)
conjugated secondary antibody (Dako, Denmark) was reconstituted at a concentration of 1:1000 in 5% blocking solution.

- anti- human β-actin (Sigma) was reconstituted at a concentration of 1:5000 in 5% blocking solution and anti-mouse HRP secondary antibody (Dako) diluted 1:1000 in 5% blocking solution.
- anti- human ubiquitin (Sigma) was reconstituted at a concentration of 1:100 in 1% blocking solution and anti-rabbit HRP secondary antibody diluted 1:1000 in 1% blocking solution.
- anti-human MAPK (active), anti SAPK (active) (Promega, Southampton, U.K) primary antibodies were reconstituted at a concentration of 1:4000 in 2% Marvel (0.1% TBS-T) and anti-rabbit HRP secondary antibody diluted 1:3000 in 2% marvel (0.1% TBS-T)
- anti- human ERK1/2 (Promega) primary antibody was reconstituted at a concentration of 1:5000 in 5% blocking solution and anti-rabbit HRP secondary antibody diluted 1:3000 in 5% blocking solution.
- anti- human JNK1/2 (Cell Signalling,) primary antibody was reconstituted at a concentration of 1:1000 dilution in 5% blocking solution and secondary HRP anti-rabbit antibody diluted 1:1000 in 5% blocking solution.
- anti- human p38 (active) (Cell Signalling) primary antibody was reconstituted at a concentration of 1:1000 in 5% BSA (0.1% TBS-T) and secondary HRP labelled anti-rabbit antibody diluted 1:2000 in 5% Marvel (0.1% TBS-T)
- anti- human p38 (Cell Signalling) primary antibody was reconstituted 1:1000 in 2% Marvel (0.1% TBS-T) and secondary HRP labelled anti-rabbit diluted 1:2000 in 2% Marvel (0.1% TBS-T)
• anti-human Galectin 1 (NovoCastra, Newcastle, U.K) primary antibody was reconstituted 1:100 in 5% BSA (0.1% TBS-T) and secondary HRP labelled anti-mouse antibody diluted 1:1000 in 5% BSA (0.1% TBS-T)

• anti-human Bcl-X\textsubscript{l} (Oncogene, U.S.A), anti-Bcl-2 (Oncogene, U.S.A) primary antibody was reconstituted 1:100 in 5% blocking solution and secondary anti-mouse HRP labelled antibody diluted 1:1000 in 5% blocking solution.

• anti-human Bcl-2 (Oncogene, U.S.A), anti-Bcl-2 (Oncogene) primary antibody was reconstituted 1:100 in 5% blocking solution and secondary anti-mouse HRP labelled antibody diluted 1:1000 in 5% blocking solution.

Secondary antibody was added to the required solution and incubated with the membrane for 1 hours and subsequently, by three 3 minute washes with 0.05% TBS-T. The membrane was incubated with the chemiluminescent solution Pierce (Pierce, Rockford, Illinois, USA) or ECL (AmershamPharmaciaBiotech, Bucks., U.K.), reconstituted as per manufacturers instructions, for 5 minutes and signals were detected via exposure to X-ray film (Agfa, Middlesex, U.K.).

2.5.2.5 Immunodetection of Translational proteins:
For the immunodetection of translation-associated proteins, the detection protocol varies slightly from the standard procedure and is outlined as follows. The membranes were incubated for 1.5 hours with the required primary antibody and then washed three times for three minutes as standard in 0.05% TBS-T (TBS containing 0.05% Tween-20). The rest of the immunodetection procedure was identical to that in 2.5.2.4.
2.5.2.6 Membrane stripping and re-probing:
In order to re-probe previously analysed membranes, a stripping procedure was employed. Membranes probed as in 2.5.2.4 were incubated for 20 minutes at 55°C in stripping buffer (62.5mM Tris, 2% SDS, pH 6.7 containing 70µl β-mercapto-ethanol per 10mls). Membranes were washed three times with TBS, followed by three 3 mins washing in 0.05% TBS-T. Three 5 minute washes with 0.05% TBS-T were carried out and when no discernable βMe odour was present, the membrane was blocked for 30 minutes in 5% blocking solution. The rest of the immuno-detection procedure was performed as in 2.5.2.4.
2.6 RNA assays:

2.6.1 RNA extraction:
Total RNA (from 1-2.5 x 10^6 cells/ml) was isolated using either TRIZOL reagent (Gibco-BRL) or the Qiagen RNeasy™ Kit (Qiagen, West Sussex, U.K.):

2.6.1.1 RNA extraction using TRIZOL Reagent:
Cells for RNA extraction (1 x 10^6) were harvested at 1400rpm for 5 minutes and 1 ml of TRIZOL reagent was added. The TRIZOL serves to disrupt the cell membrane and lysis and dissolve the cell components. 200μl of chloroform was added to allow the separation of the cellular components into DNA, RNA and proteins. The chloroform/TRIZOL mix was shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. A centrifugation step of 12,000rpm for 15 minutes at 2-8°C separates the mix into an upper aqueous layer containing the RNA and a bottom organic layer, which contains the protein. The upper layer containing the RNA was carefully removed and transferred to a clean 1.5ml tube and 0.5ml of isopropyl alcohol added. This was incubated at room temperature for 10 minutes and centrifuged at 12,000rpm for 10 minutes at 2-8°C. After this step the RNA becomes visible as a gel like pellet. This pellet was washed once with 1ml 75% ethanol, mixed, vortexed and centrifuged at 12,000rpm for 5 minutes at 2-8°C. The 75% ethanol was carefully removed from the pellet, which was air dried for 5-10 minutes. The RNA collected was reconstituted in the required amount of sterile RNase free H₂O (i.e. 87μl for the DNase reaction) and incubated at 55-60°C for 10 minutes.

2.6.1.2 RNA extraction using Rneasy™ kit:
In order to isolate total RNA using the RNeasy® kit (Qiagen), the manufacturers instructions were followed exactly. Briefly, 700μl of lysis buffer was added to a cell
pellet and this was then pipetted vigorously and placed into a QIAshredder™ column and centrifuged for 2 minutes at 8000rpm. An equal volume of 70% ethanol was added to this lysate and placed on an RNeasy spin column and centrifuged for 15 seconds at 8000rpm. The flow through was discarded and the column containing the RNA retained. 700µl of RW1 was added to this column and centrifuged. This flow through was again discarded and 500µl of Buffer RPE added and the 15 seconds centrifugation step repeated. This RPE Buffer wash was repeated and the column was centrifuged for 2 minutes until completely dry. The total RNA was eluted using the required volume of RNase free H$_2$O and centrifugation at 8000rpm for 1 minute. The RNA could then be stored at -70°C. (All buffers described above were part of the RNeasy™ kit) (Qiagen).

### 2.6.2 DNase Treatment of RNA:

RNA (isolated via TRIZOL method) was reconstituted in 100µl using 10µl of 10X DNase I buffer (40mM Tris-HCl pH 7.9, 10mM NaCl, 6mM MgCl$_2$, 10mM CaCl$_2$) plus 3µl of DNase I (10units/µl) (Roche). This reaction mix was incubated at 37°C for 30 minutes. To this mix, 100µl of phenol/chloroform/isoamylalcohol was added. This was centrifuged at 12,000rpm for 5 minutes and the upper phase transferred to a clean 1.5ml tube. The RNA was precipitated by adding 11µl LiCl and 250µl of 100% EtOH and incubating at -70°C for 20 minutes. Samples were centrifuged at 12,000rpm for 20 minutes and washed once in 70% ethanol. The RNA pellet, which should be visible, was now air dried and resuspended in 22µl DEPD-H$_2$O at 55°C for 10 minutes.
2.6.3 cDNA synthesis:
cDNA was synthesised from 24µl of reaction mix (see below for composition) plus 6µl of isolated DNase RNA (see 2.6.2 for isolation protocol). Each synthesis reaction was incubated at 37°C for 1 hour. The reaction mix for the cDNA synthesis was as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume(µl) (per reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Strand Buffer (Gibco)</td>
<td>6.0</td>
</tr>
<tr>
<td>100mM Random Hexamer (Roche)</td>
<td>2.0</td>
</tr>
<tr>
<td>4mM dNTP’s (Promega)</td>
<td>1.5</td>
</tr>
<tr>
<td>MMLV (Gibco)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.1M DTT (Gibco)</td>
<td>3.0</td>
</tr>
<tr>
<td>DMSO</td>
<td>4.5</td>
</tr>
<tr>
<td>RNasin (Promega)</td>
<td>0.6</td>
</tr>
<tr>
<td>Nuclease free H₂O</td>
<td>5.25</td>
</tr>
<tr>
<td><strong>Final Volume = 24µl + 6µl RNA</strong></td>
<td><strong>30µl</strong></td>
</tr>
</tbody>
</table>

Table 2.3: cDNA reaction mix composition
Reaction mix and volume of each constituent required for cDNA synthesis reaction in 2.6.3

2.6.4 Real Time (RQ-PCR) conditions:
Each target of interest was amplified in separate reactions i.e. one reaction mix for target of interest and one for the endogenous reference control (in this case GAPDH). Reaction mixes (25µL per reaction) contained 12.5 µl 1X TaqMan® Universal Master Mix (containing AmpiTaq Gold DNA Polymerase, AmpErase uracil-N-glycosylase (UNG)) (Applied Biosystems, Warrington, U.K.), 1µl each of both forward and reverse primers (concentrations dependent on optimisation curves), 1µl of probe
Chapter 2

(concentration dependent on optimisation curve), 2μl cDNA and 7.5μl RNase free water. The reverse transcriptase reaction was then performed as follows: 50°C for 2 mins, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute and this procedure was repeated for 40 cycles. Each reaction was set up in triplicate and also included no template controls (NTC) and no probe controls (NPC). The sequences of both primers and probes for p21₀^{BCR-ABL}, GAPDH and galectin 1 are outlined in Table 2.4.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcr-Abl:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3A2-Forward Primer</td>
<td>5'-CGTCCACTCAGCCACTGGA-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-ACTCAGACCCTGAGGCTCAAG-3'</td>
<td>85bp</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-AGCAGAGTTCAAAAGCCCTTCAGC GG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 2.4: TaqMan Primer and probe sequences** for each of the targets being examined via Taqman quantitative PCR assay (see 2.6.5 and Chapter 5) (all primers were supplied by Sigma, U.K. unless stated otherwise)

<table>
<thead>
<tr>
<th><strong>GAPDH:</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>5'-TGTTCCAATATGATTCCACCCA-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-TTGATGACAAGCTTCCCCGTTC-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-TTCCATGGGACCCTCAAGGCT-3'</td>
</tr>
<tr>
<td><strong>Galectin 1:</strong></td>
<td>80bp</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5'-TCAATCATGGGCTTGTTGCTG-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-CGCACTCGAAGGCACTCTC-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-TCGCCAGCAACCTGAATCTCAAACCT-3'</td>
</tr>
<tr>
<td></td>
<td>68bp</td>
</tr>
</tbody>
</table>

2.6.5 **TaqMan primer and probe optimisation curves:**
In order to determine the minimum concentrations of primers required giving the maximum Rn (Chapter 5), reactions were set up varying the concentrations of both forward and reverse primer from 50nM, 300nM and 900nM whilst the probe concentration was left unchanged. All reactions were performed as in 2.6.4 with the required dilutions of primers being made up in distilled water. Maintaining both forward and reverse primer at a set concentration and varying the probe concentration from 50nM, 75nM, 100nM, 125nM, 150nM, 175nM and 200nM, also determined optimal probe concentrations for each target of interest.

2.6.6 **TaqMan standard curve preparation:**
In order to accurately quantify relative gene expression, the standard curve method was employed. Standard curves of the Ct values resulting from amplification of
known amounts of the gene under investigation were constructed and from these the relative quantity of the desired gene could be determined. In this case standard curves were set up for both GAPDH and \textit{bcr-\textit{abl}}. In constructing the standard curves, the plasmid pNC210 (a gift from Dr. Nick Cross, Hammersmith Hospital, London, U.K.) containing either a segment of the GAPDH or \textit{bcr-\textit{abl}} gene was employed (constructed by Dr. Kathy Gately, St. James Hospital). From these plasmids specific quantities of either target gene were produced. Serial dilutions of each gene were then be set up which contained $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$ and 1 copy of the target gene. A quantitative PCR reaction as indicated in 2.5.4 was performed and the resulting $C_T$ values plotted versus the copy number to give a standard curve for each gene. A mathematical description in the form $y = mx + c$ was calculated where $y$ is the $C_T$ value from each reaction and $x$ is relative copy number of the gene of interest. Each $C_T$ values obtained from experimentation was analysed by the mathematical expression above and the relative copy number of the gene of interest can be calculated (Chapter 5).

\textbf{2.6.7 Statistical analysis of Taqman results:}

Results obtained for both GAPDH and \textit{bcr-\textit{abl}} by the standard curve method in 2.6.6 were used to calculate relative gene expression. This was done by normalising the target gene, in this case \textit{bcr-\textit{abl}}, against the endogenous control gene, GAPDH i.e. copy number of \textit{bcr-\textit{abl}} / copy number of GAPDH. This allows a direct comparison of values obtained in time course experiments or in experiments where different treatment protocols were examined and also allowed statistical analysis of the $C_T$'s in a comparative manner.
The comparative $C_T$ method is similar to the standard curve method except that it uses arithmetic formulas to calculate relative gene expression. The amount of target normalised to an endogenous control and relative to a calibrator is given by:

$$2^{-\Delta \Delta C_T}$$

The calibrator can be assigned as the value or reading to which all points are to be compared i.e. highest level of expression or control experiment or untreated sample.
Chapter 3

Induction of apoptosis by βGBP

in CML Cell lines
Chapter 3

Introduction:
Normal haematopoietic cell growth and development consists of highly regulated cycles of proliferation and differentiation. Even in death, the process is highly regulated. If a cell dies in a random and unregulated fashion in the process of necrosis, the final outcome can often involve localised inflammation and damage to neighbouring cells. The opposite process, apoptosis, is characterised by a series of distinct biochemical and morphological processes. Apoptotic cell death takes place in two phases: the first being the receipt of a pro-apoptotic signal with a commitment to cell death followed by an execution phase. The characteristics of apoptotic cell death are outlined in 1.5.5 e.g. condensation of the chromatin, compaction of the cellular organelles and DNA. Some of the characteristic features of apoptosis can be used as an aid in understanding the mechanisms by which cells undergo cell death. A number of techniques, which assess apoptosis both quantitatively and qualitatively, were used to assess the effects of incubating CML cell lines with βGBP.

The first technique used was the TUNEL (terminal dUTP nick end labelling) assay, which makes use of the fact that during apoptosis, the DNA is cleaved into fragments of ~200kB. Terminal deoxynucleotidyl transferase (TdT) introduces nucleotides into partially degraded DNA. Upon induction of apoptosis, DNA is cleaved and TdT adds nucleotides into these sites. Using biotin labelled dUTP and avidin labelled fluorescein isothiocyanate (FITC) molecules, the labelled cleaved DNA can be detected via flow cytometry and the levels of apoptosis assessed.

Annexin V analysis takes advantage of another characteristic event of apoptosis. During apoptosis, there is a change in the symmetry of the cell membrane molecules. This means that molecules such as phophatidylserine (PS) are “flipped” from their normal site on the inner side of the membrane to the outside of the membrane. This
PS exposure offers a binding site for molecules such as Annexin V and by linking Annexin V to fluorescent molecules such as FITC allows the apoptotic cells to be detected via flow cytometry.

Along with measuring the induction of apoptosis, the analysis of the cell cycle is also an important measure as to the status of the cell and can assess whether a cell is actively growing and dividing or is in a resting state. In addition this technique can measure the effect of drug treatments on cells and whether apoptosis is induced. Propidium iodide (PI) can bind to both DNA and RNA and can be detected using a flow cytometer. The different stages of the cell cycle contain differing amounts of DNA depending on which stage of the cycle they are e.g. cells in G2/M contain twice as much as DNA as those in G0/G1 and therefore have a characteristic cell cycle profile. Due to the fact that during apoptosis, the cellular DNA is cleaved, apoptotic cells become essentially PI negative and show a reduced fluorescence and show up as a sub-G1 population of the cells. As well as providing a profile of the cell cycle, PI staining can be used as a measure of apoptosis. Other methods of measuring the cell cycle profile of cells and the effect of various treatments on the cycle status include measuring the levels of the cell cycle control protein p27 and the use of the fluorescent molecule BrdU (bromodeoxy uridine).

A Characteristic feature of CML is the presence of the Ph+ chromosome (Rowley 1973) leading to the production of the chimeric protein p210BCR-ABL. The resulting de-regulation of the tyrosine kinase activity has been shown to be central to the pathogenesis of CML (Daley and Baltimore 1988). An important feature of p210BCR-ABL is the phosphorylation of multiple substrates (see Chapter 1 section 1.4.5), which opens up multiple signalling pathways through which enhanced signalling can occur.
and especially important are those signals involved in survival and anti-apoptotic pathways.

The resistance to apoptosis is another important feature of CML and can be attributed directly to the presence of p210^{BCR-ABL} (McGahon, Bissonnette et al. 1994). Unlike normal cells, CML cells have the ability to resist apoptosis due to growth factor withdrawal (Daley and Baltimore 1988) but also due to multiple forms of cell insult including various chemotherapeutic therapies, the presence of Fas ligand and UV irradiation (Bedi, Barber et al. 1995). For example, p210^{BCR-ABL} blocks the activation of caspase-3 (Dubrez, Eymin et al. 1998) through the abrogation of cytochrome c release from the mitochondria (Amarante-Mendes, Naekyung Kim et al. 1998).

p210^{BCR-ABL} has also been implicated in the up-regulation of pro-apoptotic proteins such as Bcl-2 (Skorski, Bellacosa et al. 1997) and Bcl-xL (Di Bacco, Keeshan et al. 2000). Other signalling pathways involved in survival signalling such as PI-3 kinase and NF-κB are also disrupted and enhanced by p210^{BCR-ABL} expression (Skorski, Kanakaraj et al. 1995; Amarante-Mendes, Naekyung Kim et al. 1998).

In addition to the ability to resist the induction of apoptosis, CML cells also have the ability to grow and proliferate without normal cellular controls hindering this proliferation. In order to successfully target and induce apoptosis in CML cells, any experimental approach has to, either directly induce apoptosis in Ph+ cells or seek to bypass the p210^{BCR-ABL} induced block on the induction of apoptosis.

This was the starting position for the studies described in this thesis. βGBP had been shown to induce significant levels of apoptosis in other cancer cell lines, including T-cell lymphomas (Novelli, Allione et al. 1999; Wells, Davies et al. 1999) and in a selective manner i.e. apoptosis was induced in malignant cells, whilst normal cells
were left unaffected. Could βGBP be used in a similar manner in the context of CML?

Could βGBP induce apoptosis in apoptosis resistant CML cells and to what degree?
Results:

3.1 Induction of Growth arrest in CML cell lines by β-Galactoside Binding Protein (βGBP):

It had been previously reported that βGBP had a growth inhibitory effect on certain cell types (Wells and Mallucci 1991). To initially examine the effect of βGBP on CML cells and establish whether βGBP had any effects on the growth characteristics of CML cell lines, cultures of CML cell lines (K562, BV173, LAMA84 and KYO-1) in exponential phase were established. These cultures were treated with 400ng/ml βGBP and monitored over a time frame from 0 to 4 days. The cell numbers were quantitated by ethidium bromide: acridine orange (EB:AO) staining in which viable cells fluoresce green under fluorescent light.

As is clearly evident in Figure 3.1, the growth of each of the four cell lines examined was significantly inhibited after treatment with βGBP. Growth of the control cultures proceeded with exponential growth being observed and this could be contrasted with the treated cell lines. There were some differences in the growth characteristics of the untreated cell lines, with K562 cell number doubling every 24 hours whilst in contrast BV173 doubles at a much slower rate. However inhibition of exponential cell growth was evident in each of the four cell lines examined after 1 day of exposure to βGBP and was maintained over each of the remaining days. The growth curve profile for each of the cell lines treated with βGBP showed that there was a complete abrogation of cell growth with only the untreated control cell lines achieving an exponential growth curve profile. Therefore βGBP treatment of a variety of CML cell lines, each with different growth characteristics, with βGBP induces significant growth inhibition.
3.2 Assessment of the levels of apoptosis induction in CML cell lines treated with βGBP via TUNEL analysis:
The assessment of growth inhibition via EB:AO staining could only indicate whether or not cell growth was inhibited. Infiltration of ethidium bromide into non-viable cells, thereby allowing these cells to fluoresce orange was not an accurate measurement of the levels of cell death or apoptosis or the stage of apoptosis being observed. As previously outlined, there are other methods that can be used to quantify or qualify the levels of apoptosis induced by a particular treatment. As part of the initial investigation to quantify accurately the levels of apoptosis, if any, being induced by βGBP, the TUNEL (terminal deoxynucleotidyl nick end labelling) assay was employed.

K562 and BV173 cells were used as they represented both of the commonest forms of the bcr-abl splice variants, namely b3a2 (K562) and b2a2 (BV173). Each cell line was treated with 400ng/ml of βGBP for time periods between 24 and 72 hours and assayed at each of these time points for apoptosis induction via the TUNEL assay. The results obtained are outlined in Figure 3.2 and 3.3. Each experiment was carried out in triplicate and an example of the kind of output from the flow cytometer is outlined in Figure 3.2, where FL1 was an arbitrary measure of the fluorescence produced by avidin-dUTP incorporation and subsequent binding of biotin-FITC. At 24 hours post treatment with βGBP, the level of apoptosis induction was minimal with no virtually no difference between treated and untreated cultures. Apoptosis induction of only 3% and 8% in K562 and BV173 respectively was detected. At 48 hours, there was a slight increase in the level of apoptosis being detected with 16% and 10% in the K562 and BV173 cultures respectively being detected. At 72 hours however, there was a large increase in the incorporation of dUTP in the treated cultures, which indicated an increase in the level of apoptosis induction in these cultures. At this time point, 54%
of the treated K562 cells and 65% of treated BV173 cells were positive for fragmented DNA.

Collating the results of the triplicate experiments produced the histograms illustrated in Figure 3.3. The mean percentage value for apoptosis induction at each time point mirror the values seen in Figure 3.2 with mean levels of apoptosis of 5.5% at 24 hours, 11.8% at 48 hours and 34.4% at 72 hours seen in treated K562 cells. In the treated BV173 cultures, mean apoptosis induction levels of 6.4% at 24 hours, 10.11% at 48 hours and 44.5% were observed. In each case, the level of apoptosis seen in the untreated control cultures remained between 5-6%, which is a level that would normally be detected seen in control cultures.

These results clearly show that βGBP had the ability to induce significant levels of apoptosis in normally apoptosis resistant CML cells and this apoptosis manifested itself at a time point approximately 48 hours after treatment with βGBP.

3.3 Cell Cycle analysis of βGBP treated CML cell lines:
As indicated in Chapter 2 (2.4.2), PI staining was carried out as part of the TUNEL assay. Both K562 and BV173 were treated with 400ng/ml of βGBP for time periods between 24 and 72 hours and assayed as outlined in Chapter 2 (2.4.2). Each experiment was carried out in triplicate and the results obtained are outlined in Table 3.1. The negative regulation of the cell cycle by βGBP has been discovered to be due to cell cycle arrest between the S and G₂ phases (Wells and Mallucci 1991). In this thesis, it was shown that treatment of CML cell lines resulted in a similar outcome. The kinds of cell cycle profiles obtained by PI staining are illustrated in Figure 3.4. Arrest of the cell cycle between the S/G₂ phases manifests itself as an increase in the percentage of cells in S phase and a decrease in the percentage of cells in G₂ phase. At
24 hours post treatment with βGBP, there was a slight increase in the percentage of cells in S phase in K562 cells, 15.22% (untreated) versus 22.26% (treated). However at 48 hours post treatment with βGBP, the cell cycle arrest becomes more pronounced in both cell lines. The S phase of K562 cells contains 16.72% when untreated but 27.30% when treated with βGBP. Comparably, BV173 cells when treated with βGBP exhibit a cell cycle arrest at the S phase with an increase in the percentage of cells in S phase from 19.85% (untreated) to 37.41% (treated). At 72 hours post treatment with βGBP, the accumulation of cells in S phase becomes less pronounced again in both cell lines however at this point, the percentage of cells in the sub-G₁ population increases. The sub-G₁ population represents cells that contain hypodiploid DNA i.e. DNA that has been cleaved during the apoptotic process. As mentioned earlier, such DNA doesn’t stain with PI and so shows up as essentially FL2 negative, where FL2 is a measure of the fluorescence due to PI. The sub-G₁ population increases from 6.70% (untreated) to 13.78% (treated) in K562 cells and from 3.31% (untreated) to 28.61% (treated) in BV173 cells. The difference between the apoptosis results obtained by the TUNEL assay (Figure 3.3) and those obtained via PI staining were due to the type of assay used. The TUNEL assay can detect large segments of DNA which have been nicked and where dUTP has been added in via the terminal transferase, whilst these sections of DNA would still be counted as normal DNA by PI staining. When the DNA has been cleaved further, it is only then that it will be detected as the sub-G₁ population via PI staining.

From these results, it was clear that similar to previous reports of βGBP activity in other cell types, treatment of CML cell lines with βGBP induced cell cycle arrest between the S and G₂ phases.
3.4 Time course of apoptosis induction in CML cell lines using Annexin V staining:

Figures 3.2 and 3.3 have established that apoptosis can be induced in the CML cell lines, K562 and BV173, following treatment with βGBP, despite the fact that these cell lines are positive for p210BCR-ABL, the presence of which can convey apoptosis resistance on these cells (Bedi, Barber et al. 1995). The level of apoptosis induced increased significantly above background control levels from 48 hours to 72 hours after addition of 400ng/ml βGBP Figure 3.2 and 3.3. To accurately determine how the apoptotic process proceeds over the time frame in which the significant levels of apoptosis are observed and also to demonstrate apoptosis induction using an alternative method, Annexin V analysis was performed. Both CML cell lines, K562 and BV173 were treated with 400ng/ml of βGBP over the time frames 36 hours to 60 hours post treatment with βGBP and Annexin V analysis of apoptosis induction performed at each time point. The binding of Annexin V FITC to externalised phosphatidylserine is an early marker of apoptosis and therefore is an important measure of the induction of apoptosis.

The results obtained from the Annexin V analysis are outlined in Figure 3.5 and clearly illustrate the progressive increase in apoptosis induction in both cell lines. There was a noticeable difference in the time course of apoptosis induction between the two cell lines. In the K562 cells, at 40 hours post treatment with βGBP there was 13.34% apoptosis compared to 9% in the untreated control but this increased to 22.08% at 44 hours with 10% in the untreated control at this point. The level of apoptosis increases up to 51.98% at 56 hours with only 5.65% in the untreated control. However BV173 cells only exhibit 8.83% apoptosis at 44 hours plus βGBP with 8.81% in the untreated control. At 56 hours this level had increased to 22.52%
and only at 60 hours post treatment does the level of apoptosis induced reach a significant level i.e. 81.1%. The significance of this difference is related to the different growth characteristics in the cell lines used (K562 versus BV173).

As seen in previous studies, βGBP has the ability to induce significant levels of apoptosis in various cancer cell lines (Wells, Davies et al. 1999) and this was confirmed here using a different assay technique. However there were subtle differences between the kinetics of apoptosis induction in the two cell lines under study.
Discussion:

As was outlined previously, the presence of the Ph’ chromosome with the associated chimeric protein, p210\textsuperscript{BCR-ABL} is a characteristic feature of CML (Rowley 1973) (Daley and Baltimore 1988) and the presence of this fusion protein can be directly responsible for most, if not all of the characteristics of CML as a disease (Daley and Baltimore 1988). These can include aberrant cell signalling, altered growth characteristics, defects in cellular adhesion and more importantly, resistance to apoptosis (see Chapter 1 section 1.5). The resistance of the CML cell to apoptosis can be directly attributed to the presence of the fusion protein p210\textsuperscript{BCR-ABL} (McGahon, Bissonnette et al. 1994; Bedi, Barber et al. 1995). This resistance to apoptosis is one of the major reasons why CML proves to be extremely difficult to treat by conventional chemotherapy. Conventional therapies for CML rely on the ability of these treatments to reduce the absolute quantity of leukaemic cells against a background of normal non-leukaemic cells. Therapies, such as busulfan or hydroxyurea, although they can induce significant levels of apoptosis can also be quite non-specific, killing normal haematopoietic cells as well as CML cells. Such treatments often have severe side effects, which nullify their benefits. Previously described treatments such as α-interferon or BMT (whether allografting or autografting), each have their own benefits and advantages but also disadvantages in the form of side effects or development of resistance to treatment. Although allogeneic BMT remains the only definitive “cure” for CML only 15-30% of patients are eligible for allogeneic SCT (for discussion of CML treatments see Chapter 1 section 1.6).

Therefore, other approaches need to be tested, ideally ones which can improve the numbers of patients suitable for therapy with minimal side effects. An important
feature of any potential new treatment is the ability to induce significant levels of apoptosis in inherently apoptotic resistant cells. Overcoming this feature of Ph' positive cells, whilst having limited toxicity in Ph' cells would provide a new approach in the treatment of CML. The compound STI571 (now known as Glivec), which has become one of the frontline therapies against CML also targets p210BCR-ABL (especially its kinase activity) directly and relies on this effect to induce significant levels of apoptosis in CML cells (see Chapter 1 section 1.6.4.1). Some examples of other experimental approaches under study include arsenic trioxide (As₂O₃) (O'Dwyer, La Rosee et al. 2002), geldanamycin (Nimmanapalli, O'Bryan et al. 2001), proteasome inhibitors e.g. PSI (Soligo, Servida et al. 2001) and butyric acid (Urbano, Koc et al. 1998). Therefore the induction of apoptosis in Ph+ cells is the central feature in these new approaches to treating CML. In this thesis, the ability of βGBP to specifically induce apoptosis in CML cells was investigated.

Initial studies of the effect of βGBP on CML cell lines focused on the effects of βGBP on the growth characteristics of these cells. CML cells are essentially growth factor independent and so independent from the normal controls on growth such as the presence of growth factors or the availability of cytokines (Daley and Baltimore 1988). Four different CML cells lines, each of which carry the p210BCR-ABL were incubated with 400ng/ml βGBP for up to four days. Growth curves, (Figure 3.1) illustrated that for each of the cell lines, a significant level of growth inhibition had occurred. In each case, cell growth in the form of exponential doubling was ablated in the treated cell lines, whilst untreated cells exhibited a normal exponential growth rate. As would be expected, there were slight differences in the growth rates for the different cell lines but as these cell lines reflect different phases of the disease and different molecular lesions, the results observed indicate that βGBP can inhibit cell
growth in CML. This complete inhibition of cell growth by βGBP had been observed in previous studies using different cell types. Mammary cells (Wells, Davies et al. 1999) and T-lymphocyte (Novelli, Allione et al. 1999) growth were both completely inhibited by incubation with βGBP in a manner similar to that observed in this study. 400ng/ml was chosen as the dose of βGBP to use following discussion with Mallucci et al and mimicking the doses of βGBP used the aforementioned previous studies on βGBP activity. However, as will be seen as this study progressed this dose level was decreased to reflect our findings (Chapter 5). However it could be concluded that incubation of growth factor independent cell (CML) lines with βGBP produced significant growth inhibition.

By using the TUNEL assay it was possible to assess the level of apoptosis that was induced by βGBP. From Figure 3.2 and 3.3, it was clear that βGBP could induce apoptosis in apoptosis resistant CML cells. The levels induced differed between the two cell lines treated but significant levels of apoptosis of 44.5% (K562) and 34.4% (BV173) were detected at 72 hours. The fragmentation of DNA is a marker of the later stages of apoptosis and these figures might not reflect the true quantity of apoptosis induced by βGBP. Annexin V binding may also be used as a measurement of the levels of apoptosis induced. As already indicated, the “flipping” of phosphatidylerine molecules to the outside membrane is an early marker of apoptosis and a comparison of the results in Figure 3.5 show that the levels of apoptosis measured by this method were higher than those detected by the TUNEL assay. In K562 cells at 56 hours, the level of apoptosis, 52%, was equal to that for 72 hours in the TUNEL assay. The results for BV173 cells are similar with 81.1% at 60 hours post addition of βGBP. These percentages for the Annexin V assay are probably a truer reflection of the degree of apoptosis induced by βGBP, with the TUNEL assay
being a quantitation of the latter stages of the apoptotic process. By using Annexin V staining, it was possible to show the time course of apoptosis induction. From Figure 3.5, it was clear that the time course of apoptosis induction in the two cell lines examined was slightly different, but with an identical outcome i.e. the induction of significant levels of apoptosis in CML cells lines. The induction of apoptosis by βGBP had also been demonstrated in other studies using other cell types (Novelli, Allione et al. 1999; Wells, Davies et al. 1999) so the results obtained here fit the profile of βGBP activity. In Figure 3.3, there was no decrease in cell number despite the fact that as shown above in Figure 3.2-3.4, there were significant levels of apoptosis induced. This apparent contradictory result may be explained by the fact that the levels of apoptosis being induced may mirror the proliferation rate of the cell lines meaning an overall static number of cells despite the induction of cell death. CML cell by their very nature proliferate rapidly and are apoptosis resistant but as seen in Figures 3.1-3, βGBP has a notable effect on the growth characteristics of these CML cell lines.

One of the characteristic features of βGBP activity is its ability to arrest the cell cycle at the S/G₂ transition (Wells and Mallucci 1991). This arrest of the cell cycle has been speculated to be one of the ways by which βGBP achieves its effect i.e. induction of apoptosis and growth inhibition, and is also one of the means by which βGBP performs one of its physiological functions i.e. the down regulation of the immune response (Blaser, Kaufmann et al. 1998). Analysis of the effect of βGBP on the cell cycle of CML cells is therefore an important feature to study as CML cells are noted for their aberrant cell cycling. p210BCR-ABL containing cells have multiple abnormalities in the cell cycle leading either to prevention of cell cycle arrest or by prevention of the down regulation of cyclin dependent kinases (Cortez, Reuther et al.
1997) or through indirectly affecting proteins which control the cell cycle e.g. E2F (Stewart, Litz-Jackson et al. 1995). After addition of βGBP and cell cycle analysis, the percentage of cells contained in the S phase increases at 48 hours post treatment and was more pronounced at 72 hours. An increase in the percentage of cells in S phase is indicative of a cell cycle block at the S/G₂ transition. Figure 3.4 and Table 3.1 illustrate this cell cycle block, which occurred in both cell lines. Another interesting feature was that at 48 hours, there was a cell cycle block but a low level of apoptosis as measured by the sub-G₁ population; however at 72 hours this sub-G₁ population increased. This observation indicates that the cell cycle block preceded the induction of apoptosis; supporting the results seen in the TUNEL and Annexin V assays. Therefore induction of the cell cycle block and the growth arrest, both precede the induction of apoptosis. This was as would be expected, as previous reports using βGBP indicate that the induction of apoptosis in other cancer cell types by βGBP was due to the conflict in cell cycle arrest and the strong mitogenic signals in cancer cells (Novelli, Allione et al. 1999). By their very nature, CML cells have strong mitogenic signals and this coupled with a cell cycle arrest and growth inhibition could be speculated to lead to the induction of the significant levels of apoptosis observed in these CML cell lines.

Therefore, βGBP had the ability to not only inhibit the growth of CML cells but also the ability to induce apoptosis in otherwise apoptosis resistant cells. The mechanism by which βGBP achieves this effect is studied in the following chapters.
Figure 3.1: βGBP inhibits the growth of CML cell lines.

CML cell lines (a) K562, (b) BV173, (c) KYO-1, (d) LAMA-84 were incubated with 400 ng/ml βGBP for 0-4 days. At each time point an aliquot of each sample was taken and cell number determined using EBAO staining. Each point represents the mean of three separate experiments +/- SEM for each set of points.
Figure 3.2: Incubation of K562 and BV173 cells with βGBP induces significant levels of apoptosis
(a) $1 \times 10^6$ cells/ml K562 or (b) $1.5 \times 10^6$ cells/ml BV173 were incubated in the presence of 400ng/ml βGBP or control diluent for time periods up to 72 hours. At each time point indicated, cells were harvested and the TUNEL assay as outlined in Figure 2.4.1 was carried out. Percentages indicated are a measure of the level of apoptosis induction. Each experiment resulted from a FACs analysis of 10,000 events and are a representative of triplicate experiments.
Figure 3.3: βGBP induces apoptosis in CML cell lines, K562 and BV173

Graphical representation of triplicate results from TUNEL assays as observed in Figure 3.2. Each histogram plot represents the mean level of apoptosis induced +/- standard deviation at each time point.
Figure 3.4: Treatment of K562 and BV173 with βGBP induces cell cycle arrest

(a) 1 x 10⁶ cells/ml K562 or (b) 1.5 x 10⁶ cells/ml BV173 were incubated in the presence of 400ng/ml βGBP or control diluent for time periods up to 72 hours. At 24, 48 and 72 hours, cells were harvested and TUNEL analysis was carried out as in 2.4.1. As part of this assay, cells were stained with Propidium iodide (PI) for cell cycle analysis. Percentages figures represent percentage of cells, which were either apoptotic (Apo) or in S phase. Each experiment resulted from a FACs analysis of 10,000 events and was a representative of triplicate experiments.
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<td>39.34 ± 8.47</td>
<td>16.72 ± 6.12</td>
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<tr>
<td></td>
<td>48+</td>
<td>9.46 ± 4.10</td>
<td>31.29 ± 15.69</td>
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<td>6.70 ± 0.97</td>
<td>40.40 ± 9.42</td>
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<td><strong>BV173</strong></td>
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<td>50.61 ± 2.67</td>
<td>17.08 ± 0.55</td>
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<tr>
<td></td>
<td>24+</td>
<td>6.65 ± 0.76</td>
<td>49.20 ± 1.96</td>
<td>14.77 ± 0.53</td>
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<td>48-</td>
<td>10.24 ± 7.62</td>
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<td>28.61 ± 9.21</td>
<td>25.21 ± 1.42</td>
<td>14.35 ± 0.76</td>
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Table 3.1: Cell cycle analysis of βGBP treated K562 and BV173 cells with βGBP
Composite analysis of percentages of either K562 or BV173 cells at each stage of the cell cycle at various time periods after βGBP treatment. Percentages were calculated from triplicate experiments as in Figure 3.4 and are mean values +/- standard deviation.
Figure 3.5: Time dependent induction of apoptosis in K562 and BV173 after treatment with βGBP

(a) 1 x 10^6 cells/ml K562 or (b) 1.5 x 10^6 cells/ml BV173 were incubated in the presence of 400ng/ml βGBP or control diluent for time periods up to 60 hours. At every four hours from 36 to 60 hours, cells were harvested and Annexin V analysis as in 2.4.2 was carried out. FACs data for 10,000 events were collected and histograms represent the mean of duplicate experiments +/- standard deviation.
Chapter 4

Examination of the effect of $\beta$GBP

on Primary mononuclear cells
Introduction:

As described in Chapter 1 (section 1.1) and illustrated in Figure 1.1, there is hierarchy of cellular differentiation with primary blasts differentiating into immature cells of a specific lineage and ultimately leading to terminal differentiation and mature cells of the many cell lineages. Primary haematopoiesis takes place in the bone marrow and associated areas and terminally differentiated and mature cells migrate from these sites to their site of activity. The premature release of immature cells into the peripheral blood system has a role to play in many of the disease associated with the haematopoietic system. For example in CML, it is the presence of Ph+ immature cells of the granulocyte lineage that is a characteristic of the disease in its initial stages followed by a move to cells of a more mature Ph+ phenotype. Haematopoiesis is controlled by regulatory molecules such as cytokines and colony stimulating factors (CSF's) and as previously described the isolation and characterisation of these factors has allowed the study of progenitor cells in vitro. By using specific factors in in vitro studies combined with short-term colony assays, the levels of specific cell types or more specifically the levels of the progenitors of this cell lineage can be assessed. Samples of bone marrow cells can be assessed for their haematopoietic potential or their ability to produce cells of a specific lineage or type. As previously mentioned, in CML there is an increase in the levels of cells of the granulocyte lineage and this would manifest itself in in-vitro assays as an increase in cells responsive to GM-CSF (granulocyte macrophage colony stimulating factor).

These progenitor cell assays are widely used in leukaemic cell studies and can allow the assessment of the leukaemic load of a sample. This is based on the observation that in many leukaemia’s, the level of progenitor cells is considerably higher than normal (Figure 4.1). As previously outlined, one of the clinical features of leukaemia
is the presence in the peripheral blood of immature or un-differentiated progenitors. This may be due to either change in cellular adhesion in the bone marrow compartment allowing the premature migration into the blood as described above or a change in the maturation rate of specific cell lineages.

Many techniques are used to study and quantify progenitor cells in different laboratories but there are common underlying characteristics. The first is the use of an optimised culture media that maximises the growth and differentiation of the cell type under study. Specific CSF’s are used in this growth media to allow the growth of specific colony types. For example GM-CSF is used to promote the growth of granulocyte and macrophage lineage cells or as they are known, colony forming units granulocyte macrophage (CFU-GM). Another essential common feature is the inclusion of a gelling agent, which means that the growth media is a semi-solid matrix.
allowing the progeny of each cell to be recognised and counted. It also allows the progeny of each progenitor cell to be recognised as a distinct colony.

The specific nature of these growth factors and the specific response they produce can be harnessed to, (1) enumerate the levels of progenitor cells and (2), monitor the response of progenitor cell growth, to exposure to chemotherapeutic agents. It was this progenitor cell culturing technique that was used in the initial part of this study.

The effect of βGBP on the growth of normal progenitor cell colonies, which are necessary for normal immune cell functioning, could also be studied using such an assay. The efficacy of any experimental approach can be measured by its ability to reduce leukemic cells in number, whilst leaving normal cells unaffected.

The effect of βGBP on the growth of CFU-GM colonies produced from cells isolated from CML patients would be an indicator of the potential of βGBP to inhibit the growth of these colonies. In CML as mentioned earlier, there is a large increase in the number of CFU-GM colonies and depending on the disease stage (whether chronic, accelerated or blast crisis) (all the CML patient samples in this study were from patients in chronic phase see Table 4.1) levels of cells of this lineage begin to accumulate and outnumber other cell types, thereby producing the disease phenotype. This is manifested in the colony assays as an increase in the number of CFU-GM colonies when compared to a non-leukemic sample.

As explained previously, the basis for therapeutic approaches in BMT and other treatments for CML is the reduction of the quantity of Ph+ cells (and therefore Ph+ CFU-GM's) whilst hopefully leaving normal haematopoietic progenitors unaffected. A number of different approaches e.g. antisense downregulation of p210^{BCR-ABL} (Wu, Joshi et al. 1995), STI571 reduction of the Ph+ CFU-GM colony number (Thiesing, Ohno-Jones et al. 2000) have used CFC assays as a measure of the effectiveness of
their anti-leukaemic potential. CFU-GM studies can be used as a model of the clonogenic potential of leukaemic progenitor cells and the effect of experimental approaches in reducing this leukemogenic potential.

In this thesis, the CFU-GM assay was used to answer similar questions. Could treating Ph+ CML cells with βGBP reduce the ability of these cells to form CFU-GM colonies and thus reduce their haematopoietic potential whilst also leaving normal cells unaffected?
Results:

4.1 To establish the linear relationship between CFU-GM number and progenitor cell number:
In order to establish that the \textit{in vitro} CFU-GM culturing technique that was being used was optimal for the growth of CFU-GM colonies from mononuclear progenitor cells, a serial dilution curve was set up. This was used to establish that the previously described linear relationship between the number of cells plated and the resulting CFU-GM colony number (Gardiner 1995) was accurate for the \textit{in vitro} assays used in this study. Mononuclear cells, isolated from a normal bone marrow sample (with consent), were serially diluted from $1 \times 10^5$ cells per plate to $1.25 \times 10^4$ cells per plate and grown using the techniques outlined in Materials and Methods 2.2.2. Each sample was plated in triplicate and the resulting colonies were counted after 14 days culturing. The results (mean of each dilution +/- SD) were outlined in Figure 4.2. An example of the type of CFU-GM colonies being counted was shown in Figure 4.3 and shows clearly shows the morphology of the CFU-GM colonies that were being assayed.

The results illustrated in Figure 4.2 clearly indicate that there was a linear relationship between the numbers of colonies obtained and the number of cells initially plated in the cell culture media. The $R^2$ value of 0.9342 indicates that there was a correlation between the two variables with no random variability. Therefore the media that was used in this study was optimal for the growth of CFU-GM colonies and illustrated the clonal nature of these types of colonies.
4.2 βGBP does not significantly affect the proliferative potential of normal hemopoietic progenitors:

The effect of βGBP, if any, on the proliferative capacity of normal cells was assayed by culturing mononuclear cells taken at harvest (with informed consent) from a number of normal bone marrow donors (n=8). The mononuclear cell fraction was isolated by density centrifugation and incubated with 400ng/ml βGBP for time periods ranging from 12 to 48 hours. In Figure 4.4, the distribution of mean inhibition of colony formation ranged from 0% to 72.6%. Table 4.1 outlines the complete data set obtained from the CFU-GM assays. The range of inhibition values for each of the eight normal donor samples, at the various time points assayed was quite large (0 – 72.6%) but as shown in Figure 4.4, results from seven out of eight donors indicated minimal effects on colony formation and the majority of the inhibition values were less than 33%. In Normal donor 4, values of 49.4% inhibition at 24 hours and 72.6% inhibition at 48 hours were determined and both these figures appear to be outlying points as compared to the rest of the inhibition values.

A clearer picture of the effect of βGBP on normal mononuclear cells was obtained through analysis of the median % inhibition results. Figure 4.5a graphs the relationship between median inhibition results for all the donor samples against the time point of analysis. These ranged from 13.4% at 12 hours, 30.8% at 24 hours and 19.5% at 48 hours. The net result was therefore that incubation of normal progenitor cells with βGBP resulted in < 30% an inhibition of the proliferative capacity of these cells.
4.3 βGBP significantly reduces the proliferative capacity of CML progenitor cells:

In order to make direct comparisons with the effect of βGBP on the proliferative capacity of CML progenitor cells, similar clonogenic assays were set up using mononuclear cells isolated from CML patients at diagnosis (n=4) with consent. Both types of clonogenic cultures were set up in identical CFU-GM media and results quantified in an identical manner after 14 days incubation. As indicated in Figure 4.4 and Table 4.1, treatment of mononuclear cells from CML patients with βGBP results in percentages of inhibition ranging from 24.2% to 77.5%. An examination of Figure 4.4 illustrated that the majority of the inhibition values are concentrated at the upper end of the percentage scale when compared to the values for the normal progenitor cells. Median inhibition values from Figure 4.5a show inhibition values of 30.4% at 12 hours, 48.2% at 24 hours and 63% at 48 hours. This means that over 30-60% of the proliferative capacity of these CML cells had been reduced through incubation with βGBP.

4.4 CFU-GM inhibition results from larger combined study:

The data presented here represents part of the data from a larger study of the effects of βGBP on CFU-GM colony formation from normal BM donors and CML patients at diagnosis section (Mc Elwaine 2000). The current data was combined with previous data allowing the effects of βGBP on normal HSCs (n=22) and CML patients at diagnosis (n=13) to be examined. Figure 4.5b illustrates the median inhibition figures for this larger combined study. The combined median inhibition values were 13.6% at 12 hours, 17% at 24 hours, 13% at 48 hours and 0% at 96 hours for the normal HSC samples and 60% at 12 hours, 48.5% at 24 hours, 53.5% at 48 hours and 63% at 96 hours for the CML samples.
4.5 Statistical analysis of inhibition percentages between normal and CML cells:
An independent student t-test with unequal variance was used for statistical analysis.
Table 4.2 shows that at each of the time points sampled, the statistical difference
between the mean inhibition values of the CML samples versus those of the normal
HSC's was statistically significant. At 12 hours, for both the values from this thesis
and those of the combined study, the probability (p value) was less than 0.01. This
places the results obtained in the 99% confidence interval meaning that the difference
between the CML and normal samples was not due to random factors and the null
hypothesis could be rejected. A similar level of confidence in the data for the 48 hour
time point was calculated (p<0.01). At 24 hours, the results from this thesis had a p
value of ≤ 0.1, whilst those in the larger combined study had a p value of ≤ 0.05
placing these values in the 95% confidence interval. Both these results were
statistically significant. Thus βGBP significantly reduced the proliferative capacity of
CML cells whilst leaving normal cells unaffected and this occurred in a statistically
significant fashion.
Discussion:

Autologous transplants exploit the ability to harvest Ph\(^-\) negative progenitors in the marrow of CML patients (Coulombel, Kalousek et al. 1983). However as explained in Chapter 1 (section 1.6.3), one of the disadvantages of autologous transplants is that there is still a significant risk of re-infusion of malignant cells, which can result in disease relapse. Therefore there are numerous studies underway to try to eliminate Ph\(^+\) cells within the autologous transplant and leave the normal Ph\(^-\) precursor cells unaffected. One approach is to use either treatments such as interferon to selectively eliminate leukaemic cells or combine mobilisation cytokines such as GM-CSF with chemotherapies to purge leukaemic cells from a background of normal cells and mobilise progenitors for reinfusion (for review of purging techniques see (Thijsen, Schuurhuis et al. 1999). In vitro purging techniques can include the use of anti-sense molecules against either \(p210^{BCR-ABL}\) or related molecules associated with the pathogenesis of CML (Wu, Joshi et al. 1995) or through the use of drugs selective in their activity against \(p210^{BCR-ABL}\) e.g. Glivec (Deininger, Goldman et al. 1997). Other means of purging Ph\(^+\) cells in an in vitro setting are the use of selection via antibody selection (antibodies against known leukaemic antigens) to exploit phenotypic differences between normal and leukaemic cells (Wognum, Krystal et al. 1992), photosensitising molecules e.g. fluorouracil (O'Brien, Kantarjian et al. 1995) and the use of hyperthermic treatments due to the differential sensitivity of leukaemic cells to cold (Douay, Lopez et al. 1986). The remaining normal cell population following a purging protocol will subsequently repopulate the marrow and re-establish normal haematopoiesis in the patient.

In order to establish the effectiveness of βGBP against CML, a differential response of normal and CML primary cells was assessed. When the results outlined above were
analysed, a significant difference between the responses of normal and CML cells to treatment with βGBP was observed. Figure 4.4 illustrates this differential effect, by plotting the percentage growth inhibition of the CFU-GM colonies against the time; at each time point a clear differential effect was evident. Normal donors show <30% inhibition, while in CML cells the degree of inhibition is significantly higher. In only one donor sample were significant levels of CFU-GM inhibition observed.

Using a larger sample size, it was evident that there is a clear difference in the response of normal cells and CML cells to βGBP. Figure 4.5b plots the median inhibition results at each time point and indicates that by increasing the sample size, only the results for 24 hours change dramatically, 30.8% (n=8) versus 16.6% (n=22). The results for 12 and 48 hours remain virtually the same no matter whether the sample size was (n=22) or (n=8), 11.8% versus 11.8% respectively at 12 hours and 11.7% and 19.6% respectively at 48 hours. Thus the inhibition values obtained at both 12 and 48 hours in this thesis (n=8) were supported by the values obtained in the larger data set. At 24 hours, the larger sample size validated the trend seen in this thesis and the results achieved statistical significance. This provides further evidence that the results obtained for normal donor 4 did not reflect the true effect of βGBP on normal haematopoietic cells. An examination of the age profile of each of the normal donors (Table 4.1) revealed an interesting trend. Donor 4 was by far the oldest donor and this sample showed the largest inhibitory effect of βGBP on normal cells. This reflected work previously reported showing differential effects of various chemotherapeutic agents on marrow cells from older patients and also illustrated the importance of patient age to the outcome of a successful BMT. This cell type seems to be susceptible to age related effects. Thus from the results of the combination of these
results with previous data, incubation of normal cells with βGBP had no major effect on the proliferative capacity of these cells.

Analysis of the CFU-GM inhibition values for the CML samples revealed that βGBP had a significant effect on the growth of CML associated colonies. The sample size was again quite small (n=4), but a clear relationship with inhibition of CFU-GM progenitors was observed (Figure 4.4, Table 4.1). Median inhibition results (Figure 4.5a) of 31.3% at 12 hours, 48.5% at 24 hours and 61.3% at 48 hours were evident meaning that >30% of the proliferative capacity of these cells was inhibited by incubation with βGBP with much higher levels of inhibition achieved at 24 and 48 hours (>45%). The results obtained at both 24 and 48 hours are of particular relevance given the observation in Chapter 3, it was observed that in CML cell lines significant levels of apoptosis were induced after 48 hours incubation with βGBP. By combining these results with those of the larger study, the sample size was again increased, (n=13) versus (n=4) and the median % inhibition results (Figure 4.5b) proved to be similar to those in this study, 60.0% at 12 hours, 48.5% at 24 hours, 53.3% at 48 hours and 62.5% at 96 hours. This study also illustrated another important feature in that each of the CML patients was in the chronic phase of the disease. This is the stage of the disease that is most amenable to treatment (see chapter 1) and the fact that this is the stage at which βGBP has a significant effect shows the potential of this molecule. Therefore it was clear from the results in this thesis and those of the combined larger study, that by treating CML cells with βGBP, a significant reduction in the proliferative capacity i.e. CFU-GM quantity, is observed.

A statistical analysis of both sets of results (this study and the combined study) revealed that the differences that were observed between the normal and CML samples were statistically significant (Table 4.2). It also established that both sets of
results obtained could be placed in a very high confidence level, a minimum of 95%, representing a statistically significant difference between the inhibitions produced by incubating normal cells with βGBP to that of incubating CML cells with βGBP. This differential effect illustrates clearly the potential of βGBP as a natural inducer of cell cycle arrest and apoptosis in CML cells, suggesting the possibility that βGBP could be useful as a purging agent. The fact that the potential purging effect of βGBP only results in a maximum of ~ 65% may seem to defeat some of the purpose to which βGBP would be aimed at. However this is a very significant amount by which CML cell growth is reduced and the potential scenario would be the use of βGBP combined with an existing chemotherapeutic agent, with both agents at a level causing very little side effects but together both causing a large additive effect. Combinative therapies are beginning to become an area of very active research and the potential for βGBP in such a scenario is an exciting prospect.

The mechanism of this differential effect on p210^{BCR-ABL} expressing cells will be investigated in subsequent chapters of this thesis.
Chapter 4

Figures:

Figure 4.2: Linear Relationship between number of Mononuclear Cells plated and numbers of resulting Colonies
Mononuclear cells were isolated from normal donor bone marrow using previously described techniques (Materials and Methods 2.3.1) and a dilution assay of differing cell numbers was set up as CFU-GM cultures as explained above (Materials and Methods 2.3.2). The resulting colonies were counted after 14 days incubation. Each figure was the result of triplicate plates and the mean figure +/- SD was plotted.

Figure 4.3: Colony Forming Unit Granulocyte/Macrophage (CFU-GM)
An example of type of CFU-GM colonies produced after 14 days culture in CFU-GM culture media as described in Materials and Methods 2.3.2 and Table 2.2. Enumeration of CFU-GM colonies was carried out at 200X magnification.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment (400 ng/ml βGBP)</th>
<th>Time Treated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>12 hours CFU-GM no. (mean)</td>
<td>24 hours CFU-GM no. (mean)</td>
</tr>
<tr>
<td></td>
<td>% inhibition</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Normal 1</td>
<td>-</td>
<td>17.0</td>
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<tr>
<td>D.O.B</td>
<td>+</td>
<td>10.6</td>
</tr>
<tr>
<td>26/9/81</td>
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<td>33.0</td>
</tr>
<tr>
<td>Normal 2</td>
<td>-</td>
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</tr>
<tr>
<td>D.O.B.</td>
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<td>8.0</td>
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<tr>
<td>2/4/55</td>
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<tr>
<td>Normal 3</td>
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<tr>
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<tr>
<td>8/17/52</td>
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<td>-</td>
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<td></td>
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<td>7.0</td>
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121
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<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>CML 1</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D.O.B</td>
<td>26/11/54</td>
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<td>Diagnosis</td>
<td>Chronic Phase</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>-</td>
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<td>15.3</td>
<td>12.0</td>
</tr>
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<td></td>
<td>+</td>
<td>5.6</td>
<td>62.6</td>
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<tr>
<td>CML 2</td>
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<td></td>
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<td></td>
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<tr>
<td>D.O.B</td>
<td>6/6/60</td>
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<tr>
<td>Diagnosis</td>
<td>Chronic Phase</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>-</td>
<td>383.6</td>
<td>381.3</td>
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<td>CML 3</td>
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<td></td>
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<tr>
<td>D.O.B</td>
<td>9/5/53</td>
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<td>Chronic Phase</td>
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</tr>
<tr>
<td></td>
<td>-</td>
<td>377.3</td>
<td>387.3</td>
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<td>+</td>
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</tr>
<tr>
<td>CML 4</td>
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<td></td>
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<td>D.O.B</td>
<td>24/1/34</td>
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<tr>
<td>Diagnosis</td>
<td>Chronic Phase</td>
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<td>22.3</td>
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<tr>
<td></td>
<td>+</td>
<td>5.0</td>
<td>77.5</td>
<td>3.6</td>
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**Table 4.1: βGBP treatment of CML and normal donor cells**

Primary mononuclear cells were isolated from CML and normal donor patients with consent as described in Materials and Methods 2.3.1. Short term cultures i.e. CFU-GM assays were set up as in Table 2.2 using cells treated with 400ng/ml βGBP or control diluent as outlined in Materials and Methods 2.3.2. After 14 days incubation, CFU-GM colony numbers were quantified and % inhibition of colony growth calculated. Table above illustrates mean colony number at each time point calculated from triplicate counts and % inhibition for each Normal donor (n=8) and each CML sample (n=4).
Figure 4.4: Effect of βGBP on normal and CML derived CFU-GM colonies

Mononuclear cells from both normal bone marrow donors and CML patients (at diagnosis) were isolated by density centrifugation (Materials and Methods 2.3.1) and treated with 400ng/ml βGBP for 12-48 hours and grown as CFU-GM cultures (Materials and Methods 2.3.2). Colonies were scored after 14 days. Graph represents % mean inhibition distribution at each time point for normal and CML cells untreated/treated with βGBP. Each point represents mean of triplicate inhibition values.
Figure 4.5: Median inhibition of βGBP on normal and CML derived CFU-GM colonies

Histograms represent % median inhibition calculated from % mean distribution values at each time point (Table 4.1) (a) median % inhibition for samples used in this thesis (b) median % inhibition for combined values from this thesis and related study (Mc Elwaine 2000).
<table>
<thead>
<tr>
<th>Sample Size</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor (n=8)</td>
<td>12 hours = 2.87</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>24 hours = 1.66</td>
<td>P &lt; 0.1</td>
</tr>
<tr>
<td></td>
<td>48 hours = 3.02</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>CML (n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor (n=22)*</td>
<td>12 hours = 4.68</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>24 hours = 2.17</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>48 hours = 5.24</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>96 hours = 5.67</td>
<td>P &lt; 0.01</td>
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<tr>
<td>CML (n=13)*</td>
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</table>

<table>
<thead>
<tr>
<th>Table 4.2: Student t-test on median % inhibition values</th>
</tr>
</thead>
<tbody>
<tr>
<td>An independent student t-test with unequal variance was carried out between mean % inhibition values for CML and Normal samples from Table 4.1. * refers to combined study with (Mc Elwaine 2000)</td>
</tr>
</tbody>
</table>
Chapter 5

The effect of βGBP on $p210^{BCR-ABL}$ expression
Introduction:

It has already been established that βGBP has the ability to induce significant levels of apoptosis in cancer cells (Wells, Davies et al. 1999) and previous work in this thesis has indicated similar effects in CML cells (Chapter 3). Significant levels of apoptosis were induced in CML cell lines, which are generally extremely resistant to apoptosis due to the presence of the p210^{BCR-ABL}. As previously outlined, the presence of the p210^{BCR-ABL} in CML cells has a significant influence on the ability of CML cells to evade chemotherapy-induced apoptosis. An elevated level of tyrosine kinase activity leads to multiple levels of survival signalling and anti-apoptotic signalling. However, as described in Chapter 3, βGBP can induce apoptosis in CML cells. Does this apoptosis induction occur in the presence or absence of p210^{BCR-ABL} expression? In order to answer this question, Western blotting and real time quantitative (RQ-PCR) of βGBP treated CML cells was performed.

5.1 Introduction to Real Time Quantitative PCR:

Standard PCR based approaches by their nature are at best semi-quantitative, as they rely on end point analysis e.g. gel detection. In order to quantify expression of a gene using a PCR based approach, a real time based PCR approach (RQ-PCR) was developed.

RQ-PCR assays are all based on the same principle pioneered by Higuchi et al (Higuchi, Dollinger et al. 1992; Higuchi, Fockler et al. 1993) in which quantitative analysis of PCR target was examined during the linear phase of the reaction and serial measurement of product accumulation was performed. As the PCR reaction proceeded, more EtBr was incorporated increasing the levels of fluorescence present as PCR product increased. The increase in fluorescence was detected and analysed by plotting increasing fluorescence versus cycle number. This produced amplification plots, which provided a measure of...
PCR product during the linear phase of the reaction. Further research on RQ-PCR has increased its practicality and established real time PCR as an extremely powerful technique for analysing changes in gene expression.

Intercalation based methods were replaced by the development of real time fluorescent probes. In RQ-PCR, in addition to forward and reverse primers, a probe is included, which contains a complementary sequence to a segment of the gene between the forward and reverse primers. These probes are labelled at the 5' end with a fluorescent reporter dye, which is linked to a quencher dye at the 3' end. In the intact state the probe emits no fluorescence signal as the reporter dye emission is quenched by the close proximity of the quencher dye. The principles of the reaction are outlined below. One type of RQ-PCR which is used here is the TaqMan assay as developed by Applied Biosystems (Warrington, U.K.). A number of fluorescent dyes are used in TaqMan assays including FAM (6-carboxyfluorescein) and VIC. Quencher dyes include TAMRA (6-carboxy-N,N,N,N-tetramethylrhodamine).

5.2 Principles of TaqMan assay:
The principles of a TaqMan reaction is illustrated in Figure 5.1. It relies on the 5'-3' exonuclease activity of Taq polymerases. If the target sequence is present, the primer and probe anneal and Taq polymerase starts to copy the template. During the extension phase, the exonuclease activity of the Taq cleaves the TaqMan probe molecule and releases the reporter dye from the quencher dye into solution. The TaqMan probe will now fluoresce if excited at the appropriate wavelength. As the PCR proceeds, fluorescent probe molecules are released into solution and the amount of fluorescence produced is directly proportional to the amount of the target sequence available. A laser achieves activation of
the probe in solution. The RQ-PCR approach is now semi-automated using machines such as 7700 DNA sequence detector (Applied Biosystems), which allows laser excitation of TaqMan probes and calculation plus analysis of fluorescence data. An advantage of this technique is that there is no signal detected from non-specific amplification because the fluorescence can only emanate where the probe binds and extension occurs.
Chapter 5

Forward Primer
5' --------------------------------► 3'

Probe
5' --------------------------------► 3'

Reverse Primer
5' --------------------------------► 3'

Polymerisation:
Reporter (R) and Quencher (Q) dyes bound to probe which binds to specific site along with forward and reverse primers.

Strand Displacement:
Reporter dye emission is quenched. Elongation of primer strands begins to displace probe from binding site.

Cleavage:
Taq DNA polymerase 5'-3' exonuclease activity cleaves reporter dye from probe and emission of fluorescence detected.

Polymerisation Completed:
Primer extension proceeds with complete displacement of probe and emission of fluorescence.

Figure 5.1 TaqMan assay
Stepwise polymerisation reaction catalysed by 5'-3' nuclease activity of AmpliTaq Gold™ DNA polymerase. (adapted from Applied Biosystems)
5.3 Analysis of TaqMan results:
The type of readout obtained from RQ-PCR is an amplification plot, which is a plot of
the fluorescence signal given as $\Delta R_n$ versus cycle number. $\Delta R_n$ is calculated by dividing
the emission intensity from the reporter dye by that of a reference dye. The reference dye
is usually ROX. An idealised amplification plot is given in Figure 5.2 and as can be seen
in the initial cycles, there is no fluorescent signal. This sets the baseline for the reaction.
As the reaction proceeds, fluorescence is detected above the baseline. This point at which
a fluorescent signal is detected is called the threshold or $C_T$ value. The reaction will then
proceed in an exponential fashion until factors such as primer concentration begin to limit
the PCR and then a plateau phase is detected usually at the end point of the reaction. $C_T$
values can be used then to determine the relative expression of a gene, as the higher the
copy number of a gene, the earlier the PCR cycle where the reaction will be detected. So
genes, which express abundant mRNA, such as those used as control genes like GAPDH
(glyceraldehyde 3 phosphate dehydrogenase) will have a low $C_T$ whilst those expressing
low amounts of mRNA will have high $C_T$ values.
Figure 5.2 Model of Real Time PCR amplification plot

Baseline is defined by threshold level and $C_T$ value is the cycle number at which a significant increase in the level of fluorescence is first detected. $\Delta R_n$ is equal to the $R_n^{+} - R_n^{-}$ and defines the amplification portion of the reaction.

Thus RQ-PCR can monitor changes in gene expression in the cell or monitor of the effects of drug treatments on gene expression profiles. In this thesis, the effect of βGBP on $p210^{bcr-abl}$ RNA expression levels was monitored.
Chapter 5

Results:

5.4 βGBP results in down regulation of p210<sup>BCR-ABL</sup> via incubation with βGBP:

In chapter 3 it was established that incubation of CML cell lines with 400ng/ml βGBP induced growth arrest and significant levels of apoptosis. CML cell lines K562 and BV173, each representing a different splice variant of p210<sup>BCR-ABL</sup> were again incubated with 400ng/ml βGBP for time points up to 48 hours, a point at which it was already established that significant levels of apoptosis had been induced (Chapter 3 Figures 3.2 and 3.5). At each time point, cells were harvested and protein extracted and Western blotting performed using an anti-abl antibody, which can detect both the p145<sup>ABL</sup> protein and the p210<sup>BCR-ABL</sup>. Figure 5.3a illustrates clearly what occurred when K562 cells were incubated with βGBP. At 24 hours post addition of βGBP, the level of the p210<sup>BCR-ABL</sup> was unchanged but at the 48 hours time point, there was a complete loss of the p210<sup>BCR-ABL</sup> protein when compared to the levels of p210<sup>BCR-ABL</sup> in the untreated control cells.

In order to establish the kinetics of p210<sup>BCR-ABL</sup> expression, a time course experiment was performed with exposure of K562 cells to βGBP at 4 hourly time points from 36 to 48 hours. Figure 5.3b indicates that at 36 hours post treatment with βGBP, there was no change in the levels of p210<sup>BCR-ABL</sup> but at 40 hours, 44 hours and 48 hours post treatment with βGBP, there was a gradual decrease in the levels of p210<sup>BCR-ABL</sup> with the complete loss of the protein at 48 hours. Therefore incubation of K562 cells with βGBP induces the down regulation of p210<sup>BCR-ABL</sup>.

In order to establish whether the results observed in K562 cells could be extrapolated to other CML cell lines, a similar time course was performed using BV173 cells. Again analysis of p210<sup>BCR-ABL</sup> levels (Figure 5.4), revealed that from 40 hours post treatment...
with βGBP, there was a down regulation of \( p210_{BCR-ABL} \) protein levels, with a virtually complete loss of this protein at 48 hours in a similar manner as to what was observed with K562 cells. In each experiment, blotting for α-actin levels was used as a positive control for protein-loading levels.

Therefore, incubation of CML cell lines with βGBP results in the down regulation of \( p210_{BCR-ABL} \) in a time dependent manner.

5.2 Different concentrations of βGBP induce identical levels of \( p210_{BCR-ABL} \) down regulation:

Figure 5.3 and 5.4 established that βGBP induced down regulation of \( p210_{BCR-ABL} \) after treatment with 400ng/ml βGBP in both CML cell lines examined. This concentration of βGBP was also observed to have a biological effect on both the induction of apoptosis (Chapter 3) and inhibition of primary CFU-GM growth in CML cells (Chapter 4). In order to establish whether there was a threshold concentration of βGBP, which still retains biological activity, a dilution curve of βGBP was performed. Western blot analysis was performed for \( p210_{BCR-ABL} \) levels and a positive result would be indicated by a decrease in \( p210_{BCR-ABL} \) levels.

The standard βGBP concentration was 400ng/ml and this was diluted to 100ng/ml and 50ng/ml using PBS and the cells treated as previously indicated. At 48 hours post addition of βGBP, a point at which down regulation of \( p210_{BCR-ABL} \) was previously observed (Figure 5.3), protein was harvested and western blotting analysis carried out. The results in Figure 5.5 indicated that for both 50 and 100ng/ml there was a complete
down regulation of \( \text{p210}^{\text{BCR-ABL}} \) levels at this time point with protein loading levels being controlled for by \( \beta \)-actin levels.

Therefore the concentration of \( \beta \text{GBP} \) needed to produce the required biological effect could be reduced by up to eight times from the original level of 400ng/ml with no disparity in the results obtained.

5.3 Different concentrations of \( \beta \text{GBP} \) induce identical levels of apoptosis:

It has been previously established that 400ng/ml \( \beta \text{GBP} \) can induce significant levels of apoptosis (Chapter 3) in CML cell lines. In order to establish whether these lower concentrations of \( \beta \text{GBP} \) could induce similar significant levels of apoptosis as those induced by 400ng/ml, the Annexin V FITC assay as outlined in Chapter 3 (Figure 3.5), was repeated. The same treatment schedule was performed but concentrations of 400, 100 and 50 ng/ml \( \beta \text{GBP} \) were used and Annexin V staining performed at 24 and 48 post treatment with \( \beta \text{GBP} \). The results obtained are presented in Figure 5.6 with a graphical representation of the levels of apoptosis induced by the differing concentrations of \( \beta \text{GBP} \) at each of the time points. It was clear from the results that there was virtually no difference between the results obtained using 400ng/ml \( \beta \text{GBP} \) and those using 100 and 50ng/ml. At 24 hours for each concentration, the results were identical. At 48 hours there were very minor differences between the 400 and 100ng/ml treatments but both were still within the standard deviation at each concentration. The result for 48 hours at 50ng/ml were also virtually identical to those for 400ng/ml and both produced over 30% apoptosis and the results were within the standard deviation for each concentration.
Therefore these different concentrations of βGBP had the ability to induce significant levels of apoptosis in a manner similar to higher concentrations of βGBP but in the case of 50ng/ml, in a manner employing eight times less βGBP.

5.4 Quantification of bcr-abl mRNA levels:
It had already been established that incubation of CML cell lines with βGBP, at concentrations from 400ng/ml to 50ng/ml, resulted in the induction of significant levels of apoptosis and the down regulation of p210^{BCR-ABL}. Because of the importance of p210^{BCR-ABL} in the pathogenesis of CML (Daley and Baltimore 1988) and the fact that p210^{BCR-ABL} alone can be sufficient in transforming cells, any approach that results in the down regulation of p210^{BCR-ABL} could be of therapeutic relevance. It was therefore important to establish the process by which p210^{BCR-ABL} down regulation occurs. Was the down regulation at the protein level or at the mRNA level? In order to answer this question, quantitative mRNA analysis was employed.

- TaqMan RT-PCR primer probe optimisation:
Initial RQ-PCR experiments were designed to optimise the primers and probe concentrations in the TaqMan reactions. The reason for optimising both primers and probes was to minimise the concentrations needed, while achieving the maximum R_\text{q.}
Any excess primer or probe could saturate and interfere with an efficient TaqMan PCR reaction. K562 cells were used and as previously mentioned these cells contain the b3a2 splice variant of p210^{BCR-ABL}. GAPDH (glyceraldehyde 3 phosphate dehydrogenase) was employed as an internal reference control gene to control for mRNA levels and to allow normalisation of the results obtained.
Primer and probe optimisation curves were set up as outlined in Materials and Methods 2.6.5. Figure 5.7 and 5.9 both illustrate the type of amplification plots obtained for b3a2 and GAPDH cDNA. An analysis of the threshold values (C_T's) in Figure 5.7b indicated that there was very little difference between the C_T values of the various forward: reverse primer combinations for b3a2. Therefore the combination of 50nM for both forward and reverse primer for b3a2 was chosen as the optimum to use in future experiments. Using the same criteria and the results in Figure 5.9b for GAPDH, the concentration of 150nM for both forward and reverse primer was chosen as that giving the maximum R_n with the minimum primer concentration.

For the probe optimisation curves, reactions were carried out as per Materials and Methods 2.6.5. Figure 5.8b gives the C_T values for the probes for b3a2 and from this, an optimum concentration of 100nM of probe was chosen. Applying the criteria of maximum R_n, minimum probe concentration to the results obtained in Figure 5.10b for GAPDH, meant that a probe concentration of 100nM was chosen as the optimum for use. Both the optimum primer and probe concentrations for both GAPDH and b3a2 were determined in this way, each giving the optimum R_n and C_T values for the concentrations chosen.

**Standard curve method of assessing down regulation of p210^{BCR-ABL}:**

In order to quantify the changes in expression of down regulation of p210^{BCR-ABL} at the mRNA level, the standard curve method was employed. Using this method (Materials and Methods 2.6.6), the relative gene expression could be determined by comparing it to standard curves prepared from known quantities of the gene. Using the plasmid pNC210, which produced known quantities of a product (in this case, both GAPDH and b3a2,
which were cloned into this plasmid see Materials and Methods 2.6.6), standard curves for both GAPDH and b3a2 were prepared and illustrated in Figure 5.12. Each standard curve produced an equation of the form \( y = mx + c \) which was used below to calculate gene copy numbers. The amplification curves obtained observed a standard exponential profile (5.11a/b) and the \( C_T \) values for each of the dilutions from \( 1 \) to \( 10^8 \) copies of each gene are outlined in Figure 5.11c.

\( 10^6 \) K562 cells were incubated with 50ng/ml βGBP for 24 and 48 hours and at each time point, RNA was extracted (Materials and Methods 2.5.1) and cDNA prepared (Materials and Methods 2.5.3). TaqMan reactions were set up in duplicate (Materials and Methods 2.6.5) for both GAPDH and b3a2 analysis and the results obtained are outlined in Figure 5.13. From the amplification plots for both b3a2 and GAPDH, there was no difference between the amplifications obtained for both the treated and untreated cells. This was borne out in the triplicate \( C_T \) values obtained at each time point for both treated and untreated K562 cells (Figure 5.13c). All of the values for b3a2 were within 0.50 of a \( C_T \) of one another. The GAPDH values were equally close, with both the untreated and treated values all within 0.80 of a \( C_T \) of one another. However, in order to accurately determine whether any down regulation of p210\(^{BCR-ABL}\) mRNA had occurred, it was necessary to use the standard curves as previously determined.

**Statistical analysis of TaqMan results:**

Using the standard curve method of quantification, the \( C_T \) values obtained for both treated and untreated cells and for both GAPDH and b3a2 in Figure 5.13c were plotted into the \( y = mx + c \) equations obtained from the standard curves in Figure 5.12. An inverse log of the values obtained from the standard curves gave a copy number for each gene at
each time point plus/minus βGBP (Figure 5.14). There was very little if any change in the copy number of \textit{bcr-abl} but there were differences in the GAPDH copy numbers between each treatment. This meant that the relative value at each time point had to be calculated for each \textit{bcr-abl} and GAPDH value. This was achieved by dividing each \textit{bcr-abl} result by the GAPDH figure and this resulted in a normalised result for each time point and treatment. As the results in Figure 5.14 indicate, the relative value showed no decrease at either 24 or 48 hours and if anything, the normalised value seemed to indicate a slightly an increased level of p210\textsuperscript{BCR-ABL} mRNA.
Discussion:

The relevance of the p210BCR-ABL in CML has already been established and discussed in detail. The de-regulation of the tyrosine kinase activity of p210BCR-ABL through changes in the folding of the chimeric protein, thereby obscuring the Abl SH3 regulatory domain (Cotter 1995), has central role in the ability of p210BCR-ABL to transform cells (Daley, Van Etten et al. 1990). This elevated and unregulated tyrosine kinase activity opens up multiple signalling pathways, which are primarily responsible for the characteristics of CML cells, namely enhanced signalling and apoptosis resistance.

Therefore the targeting of p210BCR-ABL is of prime importance in the treatment of CML. For example, Glivec, which has become an extremely successful treatment for CML, directly targets the Abl kinase activity of p210BCR-ABL (Druker, Tamura et al. 1996). Binding of ATP is blocked, inhibiting the autophosphorylation of Abl and thus preventing substrate phosphorylation. As previously explained, it is this substrate phosphorylation that plays a pivotal role in CML pathogenesis.

Treatment of CML cell lines with βGBP had already been shown to induce growth inhibition and significant levels of apoptosis (Chapter 3). The fact that these cell lines e.g. K562 and BV173, carry the p210BCR-ABL protein meant that these cell lines should be by their very nature growth factor independent and resistant to the induction of apoptosis.

Figure 5.3 illustrates the initial results obtained by treatment of K562 cells with βGBP. By treating with 400ng/ml βGBP, there was a complete loss of p210BCR-ABL at 48 hours post treatment. By using a time course of βGBP treatment, it was possible to determine the time sequence of p210BCR-ABL. At 40 hours post addition of βGBP, the levels of p210BCR-ABL begin to decrease compared to those of the untreated control and this process
proceeds until a complete down regulation was seen at 48 hours (Figure 5.3b). Therefore incubation of CML cells with βGBP induces a downregulation of \( p210^{\text{BCR-ABL}} \) protein. The identical result was obtained with BV173, which carries an alternative splice transcript of \( p210^{\text{BCR-ABL}} \) compared to K562, confirming the ability of βGBP to completely down-regulate \( p210^{\text{BCR-ABL}} \) levels regardless of the splice variant or CML cell type. By removing \( p210^{\text{BCR-ABL}} \) through down-regulation, the main reason for the abnormal phenotype of these cell types has been removed. Looking at other approaches to target \( p210^{\text{BCR-ABL}} \), molecules such as \( \text{As}_2\text{O}_3 \) (O'Dwyer, La Rosee et al. 2002) and arginine butyrate (Urbano, Koc et al. 1998) each target \( p210^{\text{BCR-ABL}} \) and induce down regulation of this protein but through different mechanisms. In both cases the induction of apoptosis is observed. The results observed in this thesis indicate that βGBP could also be placed in this category of novel anti-leukaemic agents, which can overcome the apoptosis resistance mechanisms of CML. Previous research on βGBP has established its role as a natural cytokine involved in the immune silencing phase of an immune response (Blaser, Kaufmann et al. 1998). A differential effect on cancer cells versus normal cells has also been established (Wells, Davies et al. 1999) but down regulation of an oncogene (\( p210^{\text{BCR-ABL}} \)) by treatment with bGBP has not previously been observed. However when this complete down regulation of \( p210^{\text{BCR-ABL}} \) was compared to the levels of apoptosis induced (Chapter 3), only up to a maximum of 40% inhibition was induced. The reason for this could be speculated to be due to the complexity of the signalling pathways activated through \( p210^{\text{BCR-ABL}} \). Despite the removal of the protein at the apex of these pathways through βGBP, the multitude of survival pathways activated means that the true result of \( p210^{\text{BCR-ABL}} \) downregulation and removal may not be seen until a later time.
frame. However this result shows that this protein is susceptible to down regulation and treatment with βGBP plus another apoptosis inducing agent may induce even greater levels of apoptosis at an earlier time point.

The biological activity of βGBP was established at a concentration of 400ng/ml or $10^{-8}$M (Wells and Mallucci 1991) (Novelli, Allione et al. 1999). Using a dilution curve, it was discovered that βGBP retained its biological activity down to a concentration of 50ng/ml. At both 100 and 50ng/ml, βGBP down regulated p210$^{BCR-ABL}$ (Figure 5.5) and induced the same levels of apoptosis as that induced by 400ng/ml (Figure 5.6). However there was no dose dependent effect observed at each of concentrations tested. Therefore the concentration of βGBP needed to illicit the required effect could be reduced by up to eight fold reducing the secondary effects, which may be seen at the higher concentrations.

The dose initially used was as a continuation of previous work by McElwaine et al 2000 and as used by Mallucci et al 1999 and a dilution curve carried out here probably brings the activity level of βGBP closer to its truer figure and reflects its role of a cytokine like molecule.

As already stated, treatment of CML cell lines with βGBP resulted in the down regulation of p210$^{BCR-ABL}$. It was necessary to determine whether this down-regulation was occurring at the mRNA level and preventing bcr-abl RNA transcription or was it the result of a block in the translation of bcr-abl RNA into active protein. There have been reports of chemotherapeutic agents down-regulating p210$^{BCR-ABL}$ through interference with the transcription of p210$^{BCR-ABL}$ RNA (Urbano, Koc et al. 1998). This therefore was the focus of this part of the study.
Chapter 5

Whether there was interference with RNA transcription could be answered through the use of quantitative PCR analysis. The sensitivity of this technique was clearly outlined by the standard curves (Figure 5.11) for GAPDH and \( p210^{\text{BCR-ABL}} \) transcript b3a2 where just 10 copies of each gene could be detected by this method. From the amplification plots obtained after TaqMan analysis of RNA obtained from K562 cells treated with \( \beta \text{GBP} \) (Figure 5.13), it was clear that there was no significant effect on the levels of \( p210^{\text{BCR-ABL}} \) mRNA. The \( C_T \) values for \( p210^{\text{BCR-ABL}} \) for both treated and untreated cells at both time points were all within 0.5 \( C_T \) of each other, which equates to there being no difference in the mRNA levels between samples. GAPDH was used as an internal control and to control for the levels of cDNA in each reaction. The GAPDH values however were up to 0.8 of a \( C_T \) different than one another at both 24 and 48 hours. This was quite a small difference and essentially meant that there was no difference between the treated and untreated cultures. However in order to ensure accuracy, all the results had to be normalised, meaning normalising each b3a2 value against the GAPDH value for that time point and this would equalise any random differences between experiments and allow a direct comparison of untreated and treated samples. The first part of this normalisation procedure was the determination of the total copy number for each gene and each treatment using the standard curves (Figure 5.12). As indicated in Figure 5.14, the copy number for b3a2 and therefore the \( \text{bcr-abl} \) mRNA copy number, showed no difference when compared to untreated control. The GAPDH values however seem to decrease upon treatment with \( \beta \text{GBP} \) at both 24 and 48 hours. At this point, it was necessary to normalise each \( p210^{\text{BCR-ABL}} \) value with its corresponding GAPDH value to get a relative value for each treatment. The relative \( \text{bcr-abl} \) value at both 24 and 48 hours treated with \( \beta \text{GBP} \) was...
seemed to be slightly increased which would mean that there was an increase in $p_{210}^{BCR-ABL}$ RNA. One explanation as to why this value at 48 hours appeared significant was possibly due to the fact that at 48 hours, it has been already been demonstrated that there were significant levels of apoptosis (Chapter 3). This would lead to cleavage of protein substrates, and proteins such as GAPDH, involved in metabolic processes and with short half-lives might possibly be cleaved at an earlier stage compared to proteins such as $p_{210}^{BCR-ABL}$.

In summary TaqMan analysis of $bcr-abl$ RNA expression indicated that treatment of CML cells with βGBP didn’t involve a block in transcription at the mRNA level. Thus, it was necessary to examine other means by which the observed down regulation of $p_{210}^{BCR-ABL}$ can occur and this is the focus of the next section of this study.
### Figures:

#### (a) 24h - 48h

- **βGBP (400ng/ml)**
  - 24h: +
  - 48h: +

- **p210<sup>BCR-ABL</sup>**

- **actin**

#### (b) 36h - 48h

- **βGBP (400ng/ml)**
  - 36h: -
  - 40h: +
  - 44h: -
  - 48h: +

- **p210<sup>BCR-ABL</sup>**

- **actin**

---

**Figure 5.3: βGBP down-regulates p210<sup>BCR-ABL</sup> in K562 cells.**

K562 cells (1x 10<sup>6</sup>/ml) were cultured in the presence or absence of βGBP (400ng/ml) over various time frames (a) 24-48 hours (b) 36-48 hours. At each time point, cell lysates were prepared and analysed by western blot analysis using anti-abl (1:200 dilution) and anti β-actin (1:5000 dilution) antibodies. Blots are a representative of triplicate experiments.
Chapter 5

Figure 5.4: βGBP down-regulates p210BCR-ABL in BV173 cells

BV173 cells (1.5 × 10⁶ cells/ml) were incubated in the presence/absence of 50ng/ml βGBP over the time frame 40-48 hours. Protein extracts were subjected to SDS-PAGE on a 10% gel and probed with anti-abl (1:200) and anti β-actin (1:5000) antibodies. Blots are a representative of triplicate experiments.

Figure 5.5: Differing concentrations of βGBP down-regulate p210BCR-ABL

K562 cells (1 × 10⁶ cells/ml) were incubated in the presence/absence of either 50ng/ml or 100ng/ml βGBP. After 48 hours treatment, cell lysates were prepared and protein extracts were subjected to SDS-PAGE and were probed with anti-abl (1:200) and anti β-actin (1:5000) antibodies. Blots are a representative of triplicate experiments.
Figure 5.6: Different concentrations of βGBP induce similar levels of apoptosis in K562 cells

K562 cells (1 x 10^6 cells/ml) were incubated in the presence/absence of either 4000ng/ml, 100ng/ml or 50ng/ml of βGBP. After 24 and 48 hours treatment, cells were harvested and Annexin V FITC analysis was carried out. The percentage of apoptosis induction was determined via FACS analysis. During FACS acquisition 10,000 events were collected and percentages are the mean ± SD of duplicate experiments.
Figure 5.7: Primer optimisation curves for b3a2 \textsuperscript{p210\textsuperscript{BCR-ABL}} primers

TaqMan reactions set up as outlined in Materials and Methods 2.6.4. \textsuperscript{p210\textsuperscript{BCR-ABL}} primers and probe used were outlined in Materials and Methods 2.4. Probe concentration was maintained at 100nM whilst various primer concentrations both forward and reverse were assayed. (Materials and Methods 2.5.5). (a) illustrates amplification plots for each primer set and (b) outlines \(C_T\) values for each forward/reverse primer combination.
Figure 5.8: Probe optimisation curves for b3a2 p210\textsuperscript{BCR-ABL} primers

TaqMan reactions set up as outlined in Materials and Methods 2.6.4. p210\textsuperscript{BCR-ABL} primers and probe used were outlined in Materials and Methods 2.4. Primer concentrations were maintained at optimal concentration determined as in Figure 5.4 whilst various probe concentrations were assayed. (Materials and Methods 2.5.5). (a) illustrates amplification plots for each probe concentration and (b) outlines $C_T$ values for each probe combination.
Chapter 5

(a) Amplification - gapdhprimers

(b) Primer Concentration (nM) | GAPDH C_T Values (average) | Colour Reference
--- | --- | ---
150/150 | 14.42 | ■
150/300 | 14.61 | ■
150/900 | 14.58 | ■
300/150 | 14.88 | □
300/300 | 14.86 | ■
300/900 | 14.62 | ■
900/150 | 14.39 | ■
900/300 | 14.15 | ■
900/900 | 13.80 | ■

Figure 5.9: Primer optimisation curves for GAPDH primers
TaqMan reactions set up as outlined in Materials and Methods 2.6.4. GAPDH primers and probe used were outlined in Materials and Methods 2.4. Probe concentration was maintained at 100nM whilst various primer concentrations both forward and reverse were assayed. (Materials and Methods 2.5.5). (a) illustrates amplification plots for each primer set and (b) outlines C_T values for each forward/reverse primer combination.
TaqMan reactions set up as outlined in Materials and Methods 2.6.4. GAPDH primers and probe used were outlined in Materials and Methods 2.4. Primer concentration was maintained at 100nM whilst various primer concentrations both forward and reverse were assayed. (Materials and Methods 2.5.5). (a) illustrates amplification plots for each primer set and (b) outlines $C_T$ values for each forward/reverse primer combination.
(a) Amplification - k562+gbpgapdh150;100 2+3(28-11

(b) Amplification - 24-48 (2+3) 14_11_01

(c) | K562 Cell Number | b3a2 $C_T$ Values (average) | GAPDH $C_T$ Values (average) |
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<td>$10^2$</td>
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Figure 5.11: Serial dilutions of pNC210 plasmid containing either b3a2 or GAPDH

TaqMan reactions set up as outlined in Materials and Methods 2.6.4 and 2.6.5. Primer and probe concentrations for both GAPDH and b3a2 were as determined above. cDNA was prepared from pNC plasmid (gift from Dr. Nick Cross, Hammersmith Hospital, London) containing either b3a2 and GAPDH and copy number determined and serially diluted from $10^3$ to 1 copy. (a) and (b) illustrate amplification plots for dilution sets for b3a2 and GAPDH respectively. (c) outlines $C_T$ values for serial dilutions for both b3a2 and GAPDH.
Figure 5.12: Standard curves of serial dilutions of pNC210 plasmid containing either b3a2 or GAPDH. C_t values as determined in Figure 5.9 were used to construct standard curves for both (a) b3a2 and (b) GAPDH. C_t values were plotted against log copy number and an equation of $y = mx + c$ obtained which can be used to determine copy numbers for C_t values from further experiments.
### Chapter 5

#### (a)

![Amplification - 24-48 (2+3)](file)

#### (b)

![Amplification - K562+PGBP (SOng/ml)](file)

#### (c)

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<th>K562 + βGBP (50ng/ml)</th>
<th>b3a2 C&lt;sub&gt;T&lt;/sub&gt; Values</th>
<th>GAPDH C&lt;sub&gt;T&lt;/sub&gt; Values</th>
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Figure 5.13: Analysis of gene expression by quantitative RNA analysis after treatment of K562 cells with βGBP

K562 cells were treated with 50ng/ml βGBP for 24 and 48 hours. At each time point RNA was extracted and cDNA prepared (Materials and Methods 2.6.1 and 2.6.3). TaqMan reactions for both b3a2 and GAPDH were set up (Materials and Methods 2.6.4) using previously determined primer and probe concentrations. (a) and (b) illustrate amplification curves obtained from both treated and untreated K562 cells for b3a2 and GAPDH respectively. (c) outlines triplicate C\textsubscript{T} values for duplicate experiments at 24 and 48 hours.

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<th>b3a2 Copy no. (Inv log)</th>
<th>GAPDH (from std curve)</th>
<th>GAPDH Copy no. (Inv log)</th>
<th>Relative bcr-abl (bcr-abl/GAPDH)</th>
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Figure 5.14: Determination of gene expression of b3a2 p210\(^{BCR-ABL}\) and GAPDH using standard curves

Standard curves from Figure 5.10 were used to determine gene copy number for both untreated/treated K562 cells at both 24 and 48 hours. Using the \(y=mx+c\) equations determined in Figure 5.10, the copy numbers for each of the triplicate values for duplicate experiments was determined. The relative gene expression of b3a2 for each value was obtained by dividing each b3a2 value by its GAPDH value.

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<th>GAPDH Value</th>
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Chapter 6

Effect of βGBP on downstream signalling in CML cells
Introduction:

The key event in \( p210^{BCR-ABL} \) pathogenesis, as outlined previously, is the de-regulation of the Abl kinase activity, which results in the chimeric protein having an elevated kinase activity (Lugo, Pendergast et al. 1990). It is through this elevated and unregulated tyrosine kinase activity that the transformation of CML cells can occur (Daley, Van Etten et al. 1990). Dimerization of the \( p210^{BCR-ABL} \) molecule, mediated through the NH\(_3\) domain leads to cross phosphorylation of tyrosine residues on the chimeric protein. This event along with the de-regulated Abl kinase activity results in the phosphorylation of tyrosine domains and SH2 domains. These domains, along with the other sites of activity on the chimeric protein such as sites with GEF and GAP activity (Table 1.2), present multiple binding sites for adaptor proteins and SH2 domain binding proteins (Figure 1.4). This is the central feature of \( p210^{BCR-ABL} \), an elevated tyrosine kinase activity, which opens up multiple signalling pathways through which survival signals and anti-apoptotic signals can be transduced.

Normal cell signalling through multiple signal transduction pathways allows cells to communicate extra-cellular stimuli through intracellular mechanisms. This allows cells to respond to, for example, change in extra cellular environment, stimulation by cytokines or hormones. Cell survival, growth, mitogenesis and differentiation are controlled and amended according to levels of cell signalling induced. These transduction pathways play a pivotal role in the regulation of transcription/translation-associated mechanisms. In cancer cells, the normal responses outlined above are altered and allow cancer cells to escape the control mechanisms of these signal transduction pathways, which then favour the preferential survival and proliferation of the cancer cells.
The mitogen activated protein kinase (MAPK) associated pathways are extremely important components of the signalling pathways required for the cell to respond to external stimuli. These proteins are composed of cascades of kinases, each one phosphorylating the next protein in the cascade. Activation of receptor tyrosine kinases and signalling in response to mitogenic stimuli involves this MAPK cascade and contributions from proteins such as Ras, Raf kinase and ERK1/2 (Chapter 1 section 1.5.1). Adaptor proteins such as GRB2 and SOS1 link this pathway to the receptor kinase domains. This allows the cells to regulate transcription factors such as ELK-1 and ATF2 in response to growth factor stimulation (Davis 1993). Another member of this MAPK related signalling pathways are the SAPK/JNK associated proteins. SAPK (stress associated protein kinase) unlike MAPK proteins, are activated by cellular stresses such as heat shock or irradiation (Kyriakis, Banerjee et al. 1994). JNK proteins have been associated with the induction of apoptosis but there are also reports of induction of JNK being necessary for cell survival. It has been speculated that the role of SAPK proteins in promoting apoptosis or survival depends on the extent and duration of the stimulus that is received (Roulston, Reinhard et al. 1998). The final member of this family of signalling cascades is p38, which is activated by inflammatory cytokines and environmental stresses and is associated with the induction of apoptosis (Osborn and Chambers 1996). The importance of these proteins in cell signalling in CML can be judged by the fact that many of these proteins are affected by the presence of \( p210^{\text{BCR-ABL}} \), meaning that they contribute to the ability of CML cells to resist apoptosis and proliferate without normal control mechanisms. For example autophosphorylation of Tyr177 on \( p210^{\text{BCR-ABL}} \) allows binding of GRB2, thereby recruiting molecules such as SOS1 with subsequent activation.
of Ras (Pendergast, Quilliam et al. 1993). JNK/SAPK activity has been associated with the expression of p210BCR-ABL, and was found to be necessary for the transformation of CML cells (Raitano, Halpem et al. 1995). Numerous other proteins involved in essential signal transduction and particularly in survival signalling are either up-regulated or mutated in CML cells e.g. PI-3 kinase (Skorski, Kanakaraj et al. 1995), Jak/STAT (Shuai, Halpem et al. 1996), Myc (Sawyers, Callahan et al. 1992).

Apart from defects in survival signalling, another important trait of CML cells can be attributed to altered responses to apoptotic stimuli. A characteristic feature of CML cells is their resistance to the induction of apoptosis. There are many different families of proteins involved in the apoptotic process. The apoptotic process is similar to survival pathways in that extra cellular stimuli are communicated into the cell through protein cascades and series of phosphorylation/de-phosphorylation steps. An example of the apoptotic process is outlined in Figure 1.5, as are some of the proteins involved (Chapter 1 section 1.5.5). Also, as previously described, some of these proteins are affected by the presence of p210BCR-ABL. One example is that the expression of p210BCR-ABL blocks the activation of caspase-3, which is important as an initiator of caspase activation (Dubrez, Eymin et al. 1998). Also, the release of cytochrome c from the mitochondria, which is necessary for caspase-3 activation, is blocked in CML cells (Amarante-Mendes, Naekyung Kim et al. 1998). This was speculated to be due to the expression and up-regulation of Bcl-2 (Skorski, Kanakaraj et al. 1995), an anti apoptotic protein in the same family as Bcl-XI, the expression of which is also upregulated in CML cells (Di Bacco, Keeshan et al. 2000). These proteins, along with contributions from other proteins such as
Akt (Skorski, Bellacosa et al. 1997), PI-3 kinase (Skorski, Kanakaraj et al. 1995), are integral to the mechanism of avoidance of cell death that is a characteristic of CML cells. The ultimate aim of cellular signalling is the synthesis of new proteins, which allows the cell to respond to extra cellular stimuli. The process of translation is a complex and highly regulated series of events involving proteins such as eukaryotic initiation factors (eIF's), which catalyses the assembly of a functional ribosomal complex comprising of the 40S and 60S ribosomal subunits, the mRNA and tRNA. eIF4F and p70 S6 kinase play critical roles in translation. The eIF4E complex recognises the mRNA 5' cap structure (eIF4E) plus associated factors. Figure 6.1 illustrates the complexity of protein factors involved in the translation process. Survival pathways such as Erk1/2, Akt/PKB and PI-3 kinase associated pathways, each activate and modify protein translation. One important cascade involves the activation of AKT on Ser^{473}, which activates FRAP/mTOR. This FRAP/mTOR protein kinase phosphorylates both 4E-BP1 on Thr^{37} and p70 S6 kinase on Thr^{389}. The activation of 4E-BP1 subsequently activates the eIF4E complex to initiate translation. p38 can also regulate translation by eIF4E activation, through phosphorylation of Mnk1. The activation of p70 S6 kinase leads to the activation of S6 ribosomal protein and subsequent activation of translation.
Figures 6.1: Proteins Involved in Translational process

Proteins indicated are a representative of some of the proteins involved in the complex pathways needed for protein translation.
In examining the mechanism of action of βGBP, it had been previously shown that there was a significant level of apoptosis induced by βGBP in CML cell lines (see Chapter 3). This was despite the presence of p210BCR-ABL, which is one of main perpetrators of apoptosis resistance of CML cell. It was also demonstrated that treatment with βGBP led to the down-regulation of this anti-apoptotic protein p210BCR-ABL (see Chapter 5). The next logical step therefore was to investigate the effects of this down regulation on downstream signalling in CML cells. Did down-regulation of p210BCR-ABL result in an abrogation of survival and anti-apoptotic signalling and what was the mechanism of apoptosis induction in these cells? Were there any effects on protein translation after treatment with βGBP and what was the mechanism of p210BCR-ABL down regulation given the data from the last chapter – that levels of p210BCR-ABL RNA are not affected by treatment with βGBP.
Chapter 6

Results:

6.1 Assessment of Caspase-3 activation:
Central to the apoptotic process is the activation of proteolytic enzymes such as members of the caspase family. This type of protein activation is necessary for the orderly progression of apoptosis and results in the cleavage of a wide variety of substrates e.g. actin; nuclear lamins (Blalock, Weinstein-Oppenheimer et al. 1999). Upon induction of apoptosis, the caspase cascade is assembled and activated. This involves regulatory and effector caspases such as caspase 3, which is a primary inducer of further effector caspase activation.
The quantitation of caspase activation is therefore an important measure of apoptosis induction. By using an artificial substrate containing the sequence of peptides that is the target site for caspase activity, the level of activity of a particular caspase can be assessed. In this study, the target sequence was labelled with a fluorochrome, which upon cleavage by an activated caspase can be detected by flow cytometry.
Both K562 and BV173 cell were treated with 50ng/ml βGBP for up to 72 hours. At 24, 48 and 72 hours, cells were harvested and assessed for caspase 3 activation. The level of caspase activation was compared to a positive control. The positive control involved treating Jurkat E6.1 cell with 4mM camptothecin. After treatment, 32% of the Jurkat cells contained active caspase 3. In comparison, at each of the time points examined, there was no increase in the level of active caspase 3 in both the cell lines. For example at 72 hours post treatment with βGBP, 5.4% active caspase 3 was detected (Figure 6.2) and a comparison of all time points and each cell line indicates no difference between untreated
and treated cultures. Therefore βGBP treatment of CML cell involves no activation of caspase-3.

6.2 Assessment of Proteosomal Inhibition:
Previously described work (Dou, McGuire et al. 1999) revealed that proteasome inhibition can reduce p210BCR-ABL levels and could induce apoptosis. A similar strategy was used here to assess whether βGBP was a proteosomal inhibitor. Proteosomal inhibition would manifest itself as an increase in the level of ubiquitinated proteins as the linking of chains of poly-ubiquitin to a protein mark this protein for hydrolysis by the proteasome. Any inhibition of proteasome activity therefore blocks the destruction of these marked proteins leading to an accumulation of poly-Ubq proteins. Both K562 and BV173 cells were treated with 50ng/ml βGBP for up to 48 hours. At 24 and 48 hours, protein was harvested and analysed by Western blotting using anti-ubiquitin antibodies. Treatment of K562 cells with 50µM LLnV (N-acetyl-L-leucyl-L-leucyl-norleucinal) for 24 hours served as a positive control (Dou, McGuire et al. 1999).

Figure 6.3 illustrates the outcome of the analysis for proteosomal inhibition. As indicated at both 24 and 48 hours, both treated and untreated cultures contained the same levels of ubiquitinated proteins compared to the positive control. By stripping and reprobing these blots for p210BCR-ABL, it was possible to show the down regulation of p210BCR-ABL at 48 hours had occurred as previously reported (Chapter 5). Thus down regulation of p210BCR-ABL by βGBP occurred as expected but not as a consequence of proteosomal inhibition. An identical result was obtained for BV173 cells and again stripping and reprobing the anti-ubiquitin blot for p210BCR-ABL revealed the down regulation of p210BCR-ABL at 48
hours by βGBP. Therefore the down regulation of \(p21^{\text{BCR-ABL}}\) observed after treatment with βGBP was not due to any proteosomal inhibition.

6.3 Down regulation of anti-apoptotic proteins:
The presence and activation of anti-apoptotic proteins, especially members of the BH3 family can play a role in the ability of CML cell to evade the induction of apoptosis and the levels of some of these proteins is often up-regulated in CML cells (Di Bacco, Keeshan et al. 2000). Examining the effect that βGBP treatment has on such proteins might reveal important information on how βGBP induces apoptosis in CML cells and the effects, if any, that βGBP has on downstream signalling from \(p21^{\text{BCR-ABL}}\). K562 cells were treated as previously stated i.e. 50ng/ml βGBP for up to 48 hours and then analysed for changes in expression levels of both Bcl-X\(_L\) and Bcl-2. At both 24 and 48 hours the level of Bcl-X\(_L\) was unchanged in the βGBP treated cells when compared to untreated cells (Figure 6.4). When treated K562 cells were analysed for the presence of Bcl-2, the same result was repeated. There was no difference in the levels of Bcl-2 between treated and untreated cells. Therefore incubation with βGBP resulted in no changes in the protein levels of both Bcl-2 and Bcl-X\(_L\).

6.4 Effect of βGBP on MAPK signalling pathways:
Each of the three MAPK associated pathways (MEK/ERK, MEKK/SAPK, MKK/p38) plays differing roles in the transduction of extracellular signal into intracellular responses. Due to the abnormal signalling that is evident in CML cells and the effects, which βGBP has on some of these pathways, K562 cells were exposed to 50ng/ml βGBP for up to 60
hours as previously described. Figure 6.5a illustrates the effect of βGBP on phosphorylated MAPK. After 36 hours incubation with βGBP, there was a loss of the active form of p42 MAPK (ERK1) and this loss persisted up to 48 and 60 hours. p44 (ERK2) however was unaffected in its phosphorylated state. Stripping and re-probing the blots for total MAPK protein revealed that there was no down regulation of p42 protein and although p44 was barely detectable, the levels were also unaffected (figure 6.5b). Therefore, this treatment with βGBP resulted in a loss of the phosphorylation state of p42.

An examination of the effect of βGBP on active SAPK/JNK proteins (Figure 6.6a) revealed that there was an undetectable level of active p54 (JNK2) in untreated cells, but after treatment with βGBP, there was no increase or decrease in the level of activity of p54. This was also true for p46 (JNK1), which had undetectable activity in untreated cells and remained unchanged after treatment with βGBP. After probing for total p54/p46 protein, it was revealed that neither protein was downregulated after treatment with βGBP (Figure 6.6b). There may be a slight increase in total JNK2 (p54) protein at 60 hours.

The third member of these MAPK signal transduction pathways involves the protein p38 MAPK. (Figure 6.7a) illustrates that the levels of active (phosphorylated) p38 remained unchanged after treatment with βGBP. However, when the levels of total p38 protein were assessed after treatment (Figure 6.7b), a down regulation of total p38 at both 48 and 60 hours after addition of βGBP was detected.

To ensure that the observed result i.e. down regulation of the activity of p42 ERK1 was not confined to this cell line, a similar experiment was carried out using BV173 cells.
Figure 6.5c indicates that an identical result was obtained in BV173 cells. At 36 and 48 hours, there was a decrease in the level of phosphorylated ERK1 i.e. active p42. As seen with K562 cells, there was no loss in the level of total p42 protein.

6.5 Effect of βGBP on translational proteins:
The translation of mRNA into a protein product is the final outcome for the majority of cell signalling pathways and allows a cell to respond to changes in stimuli and environmental conditions. In Chapter 5, it was established that βGBP had no effect on the levels of p210BCR-ABL mRNA transcription, so therefore it was important to establish if βGBP had an effect on the translation of the p210BCR-ABL protein. There are numerous signalling pathways leading to protein translation (Figure 6.1), thus providing many protein targets which can be assayed in order to establish if any change was occurring. The first protein assayed for was active (phosphorylated) AKT. Figure 6.8a illustrates that after treatment with 50ng/ml βGBP, there was no loss in the active form of AKT (phosphorylated on Ser473) either at 24 or 48 hours. Probing for the total protein also revealed that the expression of the Akt protein itself was not influenced by treatment with βGBP. The next target protein in the translational process to be investigated was active MNK1, which can activate the translation machinery through phosphorylation of eIF4E (Figure 6.1). Figure 6.8c indicates that treatment of K562 cells with βGBP does not result in a change in the levels of active Mnk 1 at either 24 or 48 hours.

As indicated previously, the phosphorylation of p70 S6 kinase leads to the phosphorylation of the ribosomal subunit protein S6. Phosphorylation of p70 S6 kinase on Thr389 activates this protein and stimulates its activity. Examination of the level of phosphorylation of p70 S6 kinase in K562 cells treated with βGBP (Figure 6.8b)
indicates that there was no increase/decrease in the levels of the active form of the protein at either 24 or 48 hours compared to untreated cells. S6 ribosomal protein, which is activated by phosphorylation on Ser$^{235/236}$, was the next protein in the translational machinery to be examined. Figure 6.8d revealed that there was a significant decrease in the level of active protein at 24 hours post treatment with βGBP, with a further downregulation evident at 48 hours compared to untreated control.
**Discussion:**

As previously outlined, the presence of the p210\(^{BCR-ABL}\) fusion protein can have multiple effects on cell signalling and associated pathways. Through the unregulated kinase activity of p210\(^{BCR-ABL}\) (Daley and Baltimore 1988), the activity of various different proteins associated with survival signalling and apoptosis resistance is affected. The ability of CML cells to resist apoptosis can be directly attributed to the presence of p210\(^{BCR-ABL}\) (Bedi, Zehnbauer et al. 1994) and the enhanced level of survival signalling. Therefore any treatment directed towards the treatment of CML has to overcome this resistance to apoptosis as outlined previously and also the altered cellular signalling observed in CML cells.

The induction of apoptosis had been demonstrated previously (Chapter 3) and the down regulation of the p210\(^{BCR-ABL}\) had been illustrated in Chapter 5. There were a number of questions arising from these two observations, which this section of this thesis set out to answer. The first question that was asked was the mechanism of p210\(^{BCR-ABL}\) down regulation. In Chapter 5, it was determined that transcriptional inhibition played no part in p210\(^{BCR-ABL}\) down regulation. It was then speculated that the proteasome might play a role in the observed p210\(^{BCR-ABL}\) down regulation. The proteasome is a large multicatalytic protease that regulates various cell cycle and apoptotic proteins e.g. p53, E2F.

Polyubiquitin labelled proteins mark a protein for hydrolysis by the proteasome. In the context of this study, there have been reports of proteasome inhibition resulting in the down regulation of p210\(^{BCR-ABL}\) protein and kinase activity (Dou, McGuire et al. 1999), with a resulting induction in significant levels of apoptosis. The exact mechanism of how proteasome inhibition results in p210\(^{BCR-ABL}\) down regulation is not fully understood but it has been speculated to be due to the disruption of orderly cell cycle progression and
protein function due to the accumulation of ubiquitin labelled proteins (Soligo, Servida et al. 2001). The down regulation of $p210^{BCR-ABL}$ and the appearance of apoptosis subsequent to this down regulation in these reports occurred in a similar manner as to that observed in this thesis. Upon examination of $\beta$GBP treated K562 cells however, there was no observed accumulation of ubiquitin labelled proteins, which would indicate the presence of a proteasome inhibitor (Figure 6.3). From this, it could be inferred that $\beta$GBP was not a proteasome inhibitor. Therefore, downregulation of $p210^{BCR-ABL}$ was occurring through another mechanism.

The next logical step was to examine the effect of $\beta$GBP on the translational machinery needed for efficient protein translation. Figure 6.1 illustrates the complexity of the translation process and indicates that there were a number of different protein targets, which could be evaluated as potential targets for $\beta$GBP. The active (phosphorylated) forms of AKT, MNK 1 and p70 S6 kinase were all examined and were found to be unaffected after treatment with $\beta$GBP (Figure 6.8a/b/c). However upon examination of the active form of S6 ribosomal protein, it was discovered that there was a loss in the active phosphorylated form of this protein (Figure 6.8d). Therefore $\beta$GBP was inhibiting the activation of this vital piece of the translation machinery. This results in the inhibition of translation of $p210^{BCR-ABL}$ mRNA and the net result was the down regulation of the protein, which was observed (Chapter 5). Phosphorylation of S6 ribosomal protein by p70 S6 kinase stimulates the translation of mRNA’s with 5’oligopyrimidine tracts. An examination of the mRNA sequence for $p210^{BCR-ABL}$ revealed that there was an oligopyrimidine tract in the sequence. In addition, research work by (Nimmanapalli 2001b) has indicated that down regulation of $p210^{BCR-ABL}$ by treatment with As$_2$O$_3$ results
in an inhibition of phosphorylation of p70 S6 kinase. Thus, the down regulation of p210BCR-ABL occurs through mechanisms involving βGBP interfering with protein translation.

The effects of βGBP on downstream signalling in CML cells were also investigated, as it has been previously reported that the down regulation of p210BCR-ABL itself is not sufficient to fully induce apoptosis (McGahon, Bissonnette et al. 1994) (Rowley 1996). One area, which required examination were the proteins involved in the apoptosis process. As illustrated in Figure 1.5, there are numerous proteins involved in this process and as previously outlined (Chapter 1 section 1.5.5.1) many of these proteins are affected by the presence of p210BCR-ABL. A pivotal protein required in the induction of caspase activation cascade is caspase-3. Caspase-3 acts as a primary inducer of further caspase activation and can also cleave substrates such as lamins and PARP (poly adenine diphosphosphate ADP ribose polymerase) (Amarante-Mendes, Naekyung Kim et al. 1998). However it has been shown that p210BCR-ABL expression inhibits the activation of caspase-3 and therefore blocks apoptosis, through inhibition of cytochrome c release (Amarante-Mendes, Naekyung Kim et al. 1998; Dubrez, Eymin et al. 1998). The ability of βGBP to overcome this block on apoptosis induction through the induction of caspase-3 activation was investigated. However as Figure 6.2 illustrates there was no induction of caspase-3 above that seen in the untreated control. It was clear from this result that the induction of apoptosis was occurring through a caspase-3 independent process. It has also been speculated that the ability of CML to resist the induction of apoptosis may be due to the up regulation or over expression of either Bcl-XL or Bcl-2, both anti-apoptotic proteins (Sanchez-Garcia and Grutz 1995; Amarante-Mendes, Naekyung Kim et al.
Treatment of other cell types with βGBP in previous reports has resulted in apoptosis induction of apoptosis with a decrease in the levels of Bcl-2 (Novelli, Allione et al. 1999). However an examination of the levels of both Bcl-X1 and Bcl-2 in K562 cells after treatment with βGBP induced no change in the levels of either protein (Figure 6.4). This meant that there was no increase in the levels of Bcl-2 or Bcl-X1, which would favour the induction of apoptosis over the normal ratio of pro/anti apoptotic members of the BH3 family of proteins. Therefore the induction of apoptosis in βGBP treated cells was not as a result of changes in the levels of anti-apoptotic proteins as seen in other studies.

Aberrant survival signalling plays an important role in the pathogenesis of CML (Cortez, Reuther et al. 1997). As previously mentioned, some of these pathways are directly influenced by the presence of p210BCR-ABL. Any study investigating the effect of βGBP on CML cells must therefore look at the effect of treatment on downstream signalling also. The ERK/MAPK associated pathway is linked to mitogenic stimuli and therefore survival signalling (Seger and Krebs 1995). In CML cells, this pathway is up regulated through activation of Grb2 and subsequent activation of Ras (Pendergast, Quilliam et al. 1993). Constitutive activation of Ras is one of the most important features of CML (Sawyers, McLaughlin et al. 1995) and contributes to the ability of CML cells to resist apoptosis. Examining the effect of βGBP on ERK activation in K562 cells (Figure 6.5) revealed that the levels of active p42 ERK1 were reduced from 24 hours after addition of βGBP. Thus the activity of proteins downstream from ERK1 and activity of proteins associated with this cascade such as RAS could potentially be inhibited. Inhibition of ERK proteins has been shown to induce apoptosis in CML cell lines (Kang, Yoo et al. 1998).
and it could be speculated that inhibition of the activity of ERK1 combined with a down regulation of p210$^{BCR-ABL}$ as demonstrated here may be the reason for the observed apoptosis induced by βGBP.

Associated pathways of the MAPK family such as JNK/SAPK and p38 on the other hand are associated with stress-activated pathways such as DNA damage and are involved in the induction of apoptosis (Kyriakis, Banerjee et al. 1994). Many different chemotherapeutic agents such as etoposide induce the activation of JNK proteins as part of the process by which these agents induce apoptosis (Seimiya, Mashima et al. 1997). Etoposide can also acts to induce apoptosis through inhibition of topoisomerases and p53, which explains why CML cell lines that are p53 negative, are etoposide resistant. Thus, βGBP was tested for its effects on proteins such as JNK/SAPK and p38. Figure 6.6 illustrates that there was no change in the levels of either active JNK1 or JNK2. Thus in CML cells, apoptosis induction did not involve activation of SAPK pathways. This was not unusual, as it had been reported previously by (Kang, Yoo et al. 2000) that the inhibition of ERK activity and not the activation of SAPK was necessary for apoptosis induction in K562 cells after treatment with chemotherapeutic drugs such as herbimycin. If anything there was a slight increase in the level of total p54 JNK2 at 60 hours. However this was judged not to be significant, as it was already known that significant levels of apoptosis were induced at 48 hours (Chapter 3) and an observed down regulation of p210$^{BCR-ABL}$ was already detected. Therefore the time frame of interest was from 24 to 48 hours.

However, while treatment with βGBP showed no effect on the activity of p38 i.e. phosphorylated form, there was a loss in the total amount of p38 at 48 and 60 hours post
addition of βGBP (Figure 6.7b). There may be a link between the down regulation of p38 and that of p210<sub>BCR-ABL</sub> and with the inhibition by βGBP of the translation of certain proteins. This remains to be investigated further.

However, it has been determined that not only does βGBP treatment down regulate p210<sub>BCR-ABL</sub>, but it also results in abrogation of the activity of various other proteins important for cell survival and functions which contribute to induction of apoptosis in CML cells.
Figure 6.2: Examination of the effect of βGBP on Caspase-3 activation

1 x 10^6 K562/ml or 1.5 x 10^6 BV173/ml were treated with 50ng/ml βGBP for up to 72 hours. At 24, 48 and 72 hours, cells were harvested and analysed for the activation of caspase-3 as in Materials and Methods 2.5.1. Percentages indicate the level of active caspase-3 present. Histograms are a representative of triplicate experiments and are the result of the collection of 10,000 events.
(a) PGBP (50ng/ml) 24h 48h

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βGBP (50ng/ml) 24h 48h

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Figure 6.3: Effect of βGBP on proteosomal activity in CML cell lines
(a) 1 x 10^6 K562/ml and (b) 1.5 x 10^6 BV173/ml were treated with βGBP for 24 and 48 hours. At each time point, cells were harvested and protein extracted as per Materials and Methods 2.5.2.1. Western blotting was carried out using anti-Ubiquitin (1:100 in blocking solution, Materials and Methods 2.5.2.4). Membranes were stripped and re-probed (Materials and Methods 2.5.2.6) for p210^{BCR-ABL} expression using anti-abl (1:200 in blocking solution, Materials and Methods 2.5.2.4). Probing for β-actin controlled for equal protein loading. Blots are representative of triplicate experiments.
Figure 6.4: Effect of βGBP treatment on apoptotic associated proteins

K562 cells (1 x 10^6 cells/ml) were treated with βGBP (50ng/ml) for times periods up to 60 hours. At 36, 48 and 60 hours cells were harvested and protein extracted (Materials and Methods 2.5.2.1). Western blots were probed for either (a) Bcl-2 (1:100 in blocking solution) or (b) Bcl-X_L (1:100 in blocking solution, Materials and Methods 2.5.2.4). Probing for β-actin controlled for equal protein loading and blots are a representative of triplicate experiments.
Figure 6.5: Effect of βGBP on the activity of MAPK ERK1/2

K562 cells (1 x 10⁶ cells/ml) and BV173 (1.5 x 10⁵ cells/ml) were treated with βGBP (50ng/ml) for times periods up to 60 hours. Protein was extracted at 36, 48 and 60 hours and Western blotting carried out (Materials and Methods 2.5.2). Blots were probed for (a) active (phosphorylated) MAPK ERK1/2 in K562 cells, (b) total ERK1/2 in K562 cells and (c) active MAPK, total ERK1/2 in BV173 cells. Secondary protein binding controlled for protein loading.
Figure 6.6: Effect of βGBP on the activity of SAPK/JNK 1/2

K562 cells (1 x 10^6 cells/ml) were treated with βGBP (50ng/ml) for times periods up to 60 hours. Protein was extracted at 36, 48 and 60 hours and Western blotting carried out (Materials and Methods 2.5.2). Blots were probed for (a) active (phosphorylated) SAPK/JNK1/2 and (b) total JNK1/2. Secondary protein binding controlled for protein loading.

Figure 6.7: Effect of βGBP on the activity of p38 MAPK

K562 cells (1 x 10^6 cells/ml) were treated with βGBP (50ng/ml) for times periods up to 60 hours. Protein was extracted at 36, 48 and 60 hours and Western blotting carried out (Materials and Methods 2.5.2). Blots were probed for (a) active (phosphorylated) p38 MAPK and (b) total p38 protein. Secondary protein binding controlled for protein loading.
Figure 6.8: Effect of βGBP on the activity of translation associated proteins

K562 cells (1 x 10^5 cells/ml) were treated with βGBP (50ng/ml) for times periods up to 48 hours. Protein was extracted at 24 and 48 hours and Western blotting carried out (Materials and Methods 2.5.2). Blots were probed for (a) active Akt (upper blot) and total Akt (lower blot), (b) active p70 S6 kinase, (c) active phospho-Mnk1, (d) active S6 ribosomal protein. Secondary protein binding controlled for equal protein loading.
Discussion
The nature of CML as a disease is as a malignant clonal haematopoietic stem cell disorder affecting cells of the granulocytic lineage. The main characteristic of CML is the presence of the Ph' chromosome, with the resulting formation of a chimeric protein, p210_{BCR-ABL} (Ben-Neriah, Daley et al. 1986). The presence of p210_{BCR-ABL} is regarded as the primary event in CML pathogenesis as demonstrated by the experiment whereby murine cells transduced with p210_{BCR-ABL} were transformed and a leukaemia like disease resulted (Daley, Van Etten et al. 1990). As previously explained, p210_{BCR-ABL} is a constitutively active tyrosine kinase (Lugo, Pendergast et al. 1990) and this unregulated and enhanced tyrosine kinase activity was found to be essential for the transforming ability of p210_{BCR-ABL} (Daley and Baltimore 1988). The autophosphorylation of p210_{BCR-ABL} (Reuther, Fu et al. 1994) creates multiple binding sites for adaptor proteins thereby multiplying the signalling pathways affected by p210_{BCR-ABL}. The aberrant nature of p210_{BCR-ABL} and the enhanced tyrosine kinase activity of the protein affect many different downstream signalling pathways.

A number of proteins in an array of different signalling pathways are affected in CML and include Ras (Sawyers, McLaughlin et al. 1995), PI-3 kinase (Skorski, Kanakaraj et al. 1995), JAK/STAT (Shuai, Halpem et al. 1996) and Myc (Sawyers, Callahan et al. 1992). The upregulation of the Ras pathways mediated through Tyr177 and Grb2 binding (Pendergast, Quilliam et al. 1993) plays a pivotal role in survival signalling and many different proteins such as ERK/MAPK interact with activated Ras to amplify these survival signals. The enhanced tyrosine kinase activity also has a role to play in the aberrant cell growth, adhesion and apoptotic pathways (Chapter 1 section 1.5) associated with CML. For example, Raf, which is downstream of Ras, is speculated to phosphorylate the pro-apoptotic protein Bad blocking its activity (Zha, Harada et al. 1996) and also there are reports that Ras phosphorylates the anti-
apoptotic protein Bcl-2 on Ser\textsuperscript{70} promoting its activity (Ito, Deng et al. 1997). The enhanced activity of proteins such as PI-3 kinase in Ph\textsuperscript{+} positive cells, has been associated with the up-regulation of the anti-apoptotic protein Bcl-2 (Sanchez-Garcia and Martin-Zanca 1997; Skorski, Bellacosa et al. 1997) and this results in the blocking of the release of cytochrome c, which is essential for the activation of caspases, especially caspase-3 (Amarante-Mendes, Naekyung Kim et al. 1998). The expression of other members of the BH3 family of anti-apoptotic proteins such as Bcl-X\textsubscript{L}, has also been found to be increased in p210\textsuperscript{BCR-ABL} containing cells (Di Bacco, Keeshan et al. 2000). STAT proteins have also been found to transcriptionally activate Bcl-X\textsubscript{L} (Horita, Andreu et al. 2000) and as seen previously STAT family members can bind to autophosphorylated p210\textsuperscript{BCR-ABL} through adaptor proteins bound and be phosphorylated by active p210\textsuperscript{BCR-ABL}.

The net result of the aberrant signalling in Ph\textsuperscript{+} cells is apoptosis resistant cells, which have been shown to resistant to growth factor deprivation, chemotherapeutic drugs, Fas ligand and UV irradiation (Bedi, Barber et al. 1995). Existing treatment strategies against CML have a measure of success e.g. a “cure” rate of 50 –60% for allogeneic SCT (stem cell transplant) (Sawyers 1999), however there are problems with eligibility for an allo SCT such as age, absence of a HLA compatible donor, disease stage and this means that only 15-30% of patients are eligible for allo SCT (Chapter 1 section 1.6.3). Certain chemotherapeutic approaches have been successful in treating CML e.g. α-interferon, which can be a successful treatment but is compromised in its effectiveness in small numbers of patients due to development of resistance and toxicity (Sawyers 1999). Given the need for therapeutic approaches for patients who cannot benefit from standard chemotherapy or allogeneic transplantation, a number of
approaches have been developed which target the $p210^{BCR-ABL}$ protein and its tyrosine kinase activity.

The direct targeting of $p210^{BCR-ABL}$ benefits from the fact that, this fusion protein is only present in CML cells, and is essential for the transformation of these cells. An example of this targeted therapy is the anti-CML drug Glivec (STI571), which targets the binding of ATP to $p210^{BCR-ABL}$ and thus prevents the activation of the tyrosine kinase activity (Druker, Tamura et al. 1996). It has proven to be a very successful treatment with a high success rate e.g. a complete haematological response was seen in 91% of chronic phase patients and a major cytogenetic response was seen in 55% of these cases (Druker, Sawyers et al. 2001)

We decided to investigate the activity of the molecule β Galactoside Binding Protein (βGBP) and its potential for the induction of apoptosis in CML. Previous investigation of the activity of βGBP has shown that treatment of cancer cells with βGBP resulted in significant levels of apoptosis being induced whilst normal cells were unaffected (Novelli, Allione et al. 1999; Wells, Davies et al. 1999). βGBP results in a cell cycle block at the S/G2 transition (Wells and Mallucci 1991), which may contribute to the effects observed.

Results on cancer cells were all based on studies in cell lines, however it was important to establish the effects of any new agent on primary patients cells also. In haematopoietic malignancies, the use of short-term colony assays allows experimental evaluation of the effects of an agent such as βGBP on primary cells. Thus cells were collected from CML patients at diagnosis and from normal donors for allo SCT.

The results outlined in Chapter 4, indicated that after treatment with βGBP, there was a differential effect between the levels of CFU-GM growth inhibition in CML primary cells compared to the normal haematopoietic allo-SCT donor. A comparison of the
median growth inhibition at each of the time points of analysis (Figure 4.5a) revealed that the inhibition of CML colony growth at 12 and 48 hours was more than three fold higher than those observed in normal haematopoietic cells. When the results from this thesis were combined with those from the larger study (Figure 4.5b), the combined sample confirmed the differential effect of βGBP on CML cells as compared with normal haematopoietic cells, as a larger sample size in these kinds of studies gives a more valid quantitation of results.

The results obtained from short-term cultures were promising, particularly if βGBP was being considered as a potential “purging” agent. An important observation was that each of the CML patients examined in this study and the majority of the combined study were in chronic phase. This is the earliest stage of the disease and the stage at which treatments can have the greatest effects due to the amenable nature of the disease at this stage. By showing such a significant effect at the chronic phase βGBP joins panoply of novel treatments, which could have a significant effect if used in conjunction with each other and combined with the new methods of early diagnosis. But there also needs to be an examination of the effect of βGBP on the more resistant phases of CML, blast crisis and accelerated phase. A examination of CML patient samples at these stages could reveal a truer indication of the potential of βGBP as an anti-CML agent. However, before any other assumptions about the activity of βGBP could be made, it was necessary to investigate further the mechanisms of how βGBP induces apoptosis in CML cells.

The induction of apoptosis is a central feature of any novel approach aimed at killing CML cells. This is primarily due to the ability of Ph+ cells to resist the induction of apoptosis by numerous chemotherapeutic agents e.g. camptothecin, etoposide, staurosporine, (Kang, Yoo et al. 2000). The ability to resist the induction of apoptosis
is primarily due to the enhanced signalling and tyrosine kinase properties of the fusion protein which results in aberrant survival signalling and changes in the functioning of apoptosis related proteins (Chapter 1 section 1.5). A feature of CML is the accumulation of leukaemic blasts, which rather than an increase in the level of proliferation, is speculated to be due to an inhibition of apoptosis (Rowley, Keng et al. 1996)(Rowley 1996 keng). Therefore there appears to be a continuous block on the induction of apoptosis and this is achieved at a number of sites due to the interaction of p210<sup>BCR-ABL</sup> with various proteins and signalling cascades e.g. expression of p210<sup>BCR-ABL</sup> blocks the activation of caspase-3 (Amarante-Mendes, Nackyung Kim et al. 1998). An initial examination of the effect of βGBP on the growth of various CML cell line (Figure 3.1) revealed that the growth of each cell lines was almost completely inhibited after treatment with βGBP. This result was not unexpected, as previous work with βGBP on other cell types, had also revealed that growth inhibition was one of the characteristics of the activity of βGBP (Blaser, Kaufmann et al. 1998; Wells, Davies et al. 1999). Given that one of the modes of activity of βGBP is the induction of cell cycle arrest at S/G<sub>2</sub> (Wells and Mallucci 1991), it was not surprising that there was an induction in growth arrest in the cells lines examined here, as an induction of cell cycle arrest by whatever means will result in growth inhibition.

Along with the initiation of growth inhibition, it was necessary to examine whether there was any induction of apoptosis. These CML cell lines by their nature are apoptosis resistant due to the expression of p210<sup>BCR-ABL</sup>, so by using different methods for the quantitation of apoptosis, the levels of apoptosis induced, if any, were analysed. A consequence of using the TUNEL assay was that the cell cycle status could also be assessed along with the levels of apoptosis. In both of the CML cell lines examined, there was an increase in the percentage of cells in S phase indicating
the presence of a cell cycle block at the S/G2 transition (Table 3.1). This observation correlates with the site of activity of βGBP in other cell types (Wells, Davies et al. 1999). However, this induced cell cycle block might also be important for the effect of βGBP on CML cells because one of the features of CML cells is that in Ph+ cells, the cell cycle can be deregulated due to the effect that the expression of p210BCR-ABL has on important proteins involved in cell cycle regulation e.g. p210BCR-ABL prevents the down regulation of cdk proteins and therefore blocks induction of cell cycle arrest (Cortez, Reuther et al. 1997). p210BCR-ABL also down regulates the cell cycle control protein p27, through its activation of PI-3 kinase/AKT and therefore these Ph+ cells have no checkpoint controls and cycle without inhibition (Gesbert, Sellers et al. 2000). It could that by inducing a cell cycle block in cells that are normally cycling without controls may predispose βGBP treated cells towards apoptosis. When the levels of apoptosis were quantitated using the TUNEL and Annexin V assay, it was found that there were significant levels of apoptosis induced after treatment with βGBP (Figures 3.2, 3.3 and 3.5). The induction of apoptosis proceeded in a time dependent fashion from 56 hours after addition of βGBP. Therefore treatment of CML cell lines (BV173 and K562) with βGBP had the ability to induce apoptosis. The induction of apoptosis by βGBP has been previously observed in various other cell types e.g. antigen activated T-cells (Blaser, Kaufmann et al. 1998), mammary cell (Wells, Davies et al. 1999), but here apoptosis was demonstrated in a cell type which had previously been shown to be apoptosis resistant (McGahon, Bissonnette et al. 1994). The induction of apoptosis in CML cells is the main aim of most of the novel approaches under study e.g. arsenic trioxide (As2O3) (O'Dwyer, La Rosee et al. 2002), geldanamycin (Nimmanapalli, O'Bryan et al. 2001), so βGBP has already exhibited an important trait which merits further study.
In the initial section of this study, the concentration of βGBP used in experiments was 400ng/ml. This concentration was derived from previous studies in this lab and from communication with other research groups working on βGBP e.g. (Wells, Davies et al. 1999). However it was decided to examine (upon consultation with collaborating groups) whether there was an optimal concentration of βGBP. A dilution curve with various concentrations was set up and it was determined that a concentration of 50ng/ml gave identical effects i.e. induction of significant levels of apoptosis to that achieved with 400ng/ml (Figure 5.3 and 5.4). This meant that eight times less βGBP was required to elicit a biological effect, demonstrating its powerful anti-leukaemic effect and also potentially allowing fewer side effects to occur. Another study has determined the activity of Galectin-1 (the dimeric form of βGBP) to be at the minimum, 50μg/ml (Kopitz, von Reitzenstein et al. 2001). This was interesting because the observed activity of βGBP was 50ng/ml, which indicated that the biologically active concentration of βGBP, as observed in this study was a thousand fold less than that of the related lectin, Galectin-1. In order to fully determine what the optimum concentration of βGBP for use is, it would be important to set up a complete dose response curve to dilute out the effect of βGBP fully and determine at what dose level βGBP still retains its cytokine like activity.

The contributing role of p210BCR-ABL expression to apoptosis resistance prompted examination of the effect of βGBP on p210BCR-ABL expression. Treatment of CML cell lines, both K562 and BV173, with βGBP resulted in the downregulation of the p210BCR-ABL protein (Figure 5.1 and 5.2). The down regulation occurred in a time dependent fashion beginning at 40 hours post addition of βGBP. The expression of p210BCR-ABL is widely regarded as the pivotal event in the transformation of CML
cells (Daley and Baltimore 1988) and the expression of the fusion protein with its elevated tyrosine kinase activity is responsible for the aberrant phenotype of CML cells (Lugo, Pendergast et al. 1990). Therefore any loss of this protein through down regulation should confer susceptibility on these cells to the induction of apoptosis. The time course of this downregulation was important as it was necessary to determine whether the observed down regulation was the primary event preceding the induction of apoptosis or was this protein down regulation, a consequence of the apoptotic process? From the time course experiments, it was clear that the downregulation of \( p210^{\text{BCR-ABL}} \) preceded the induction of apoptosis. At 40–44 hours post addition of \( \beta \)-GBP, the levels of \( p210^{\text{BCR-ABL}} \) begin to decrease with a complete loss of the protein at 50 hours (Figure 5.1b). When the levels of apoptosis were analysed, there was no significant apoptosis induced until after 48 hours in both cell lines examined. This meant that the loss of the \( p210^{\text{BCR-ABL}} \) protein was associated with the induction of significant levels of apoptosis. Novel compounds under study against CML such as \( \text{As}_2\text{O}_3 \) (O'Dwyer, La Rosee et al. 2002) and geldanamycin (Nimmanapalli, O'Bryan et al. 2001) both lead to downregulation of \( p210^{\text{BCR-ABL}} \) prior to the induction of apoptosis.

The next logical step was to determine the site at which the downregulation of \( p210^{\text{BCR-ABL}} \) occurs. Did \( \beta \)-GBP treatment result in a block in the transcription of the \( p210^{\text{BCR-ABL}} \) gene or was the downregulation occurring at the translational level. By utilising the TaqMan quantitative PCR assay, it was determined that there was no loss in the levels of \( \text{bcr-abl} \) mRNA at either 24 or 48 hours (Figure 5.13). From an analysis of mRNA levels therefore, it was clear that the down regulation of \( p210^{\text{BCR-ABL}} \) was not occurring at the level of transcription. Targeting the transcription of \( \text{bcr-abl} \) mRNA has already been shown to be the mechanism by, which some targeted
novel therapies function i.e. arginine butyrate (Urbano, Koc et al. 1998) but it was not the case for βGBP.

These results led to the investigation of the effect of βGBP on translational mechanisms needed for the translation of bcr-abl mRNA into protein. The translation process as indicated in Figure 6.1, is a complex and tightly controlled process-involving numerous proteins and cascades of activation and phosphorylation. Upon examination of the proteins involved in this translation process, it was discovered that the activation of one specific member of this process was prevented in cells treated with βGBP, namely the activation of S6 ribosomal protein (Figure 6.8). S6 ribosomal protein is activated by p70 S6 kinase and is involved in the translation of proteins with 5' oligopyrimidine tracts and indeed this kind of nucleotide tract exists in the mRNA for p210BCR-ABL. Therefore the inhibition of the activation of S6 ribosomal protein blocks the translation process and specifically the translation of p210BCR-ABL. This results in the observed down regulation of p210BCR-ABL. This down regulation of p210BCR-ABL through inhibition of the translation process has been previously observed. Arsenic trioxide (As2O3), which also down regulates p210BCR-ABL and induces significant levels of apoptosis in CML cells (O'Dwyer, La Rosee et al. 2002) in a similar fashion to that observed with βGBP, was also found to inhibit the activation of proteins involved in translation. More specifically, As2O3 prevented activation of p70 S6 kinase and this protein was a part of the same protein cascade affected by βGBP (Nimmanapalli 2001b). Therefore this showed that the site of activity of βGBP was not unique to this protein and may represent a site of vulnerability in CML cells. In a recently published study, it has been shown there is constitutive phosphorylation of S6 ribosomal protein and 4E-BP1 in CML cells and this may suggest that p210BCR-ABL regulates translation of proteins via these
translational pathways (Ly 2003). As already described, these translational proteins regulate the translation of p210\(^{\text{BCR-ABL}}\) itself, meaning that in CML cells, p210\(^{\text{BCR-ABL}}\) could regulate its own translation. This report also suggests that these translation proteins represent a potential site of vulnerability and inhibitors of the translational process could be used to target this vulnerability and as demonstrated in this thesis, a candidate protein could be \(\beta\)GBP.

However, it has been previously reported that targeting p210\(^{\text{BCR-ABL}}\) alone was not sufficient to induce apoptosis but only rendered these cells more susceptible to the induction of apoptosis due to other insults such as chemotherapeutic agents (McGahon, Bissonnette et al. 1994). This would seem to be a logical observation because expression of p210\(^{\text{BCR-ABL}}\) alone or transfection of cells with p210\(^{\text{BCR-ABL}}\) renders these cells resistant to apoptosis through many different means (Daley, Van Etten et al. 1990; Bedi, Barber et al. 1995), so by removing this block on apoptosis, it should be possible to induce apoptosis using conventional chemotherapies. Therefore, it was necessary to examine whether \(\beta\)GBP has other effects on CML cells as well as the down regulation of p210\(^{\text{BCR-ABL}}\). The down regulation of p210\(^{\text{BCR-ABL}}\) could be viewed as the primary effect but were there secondary effects mediated by \(\beta\)GBP necessary for the induction of apoptosis?

Various downstream signalling proteins and proteins involved in the induction of apoptosis were examined. Many of the proteins examined are involved in survival signalling in CML cell and the ability of CML cells to resist apoptosis. Expression of the anti-apoptotic proteins, Bel-X\(_L\) and Bel-2 are up regulated in CML cells and contribute to the resistance of CML to apoptosis (Di Bacco, Keeshan et al. 2000) (Skorski, Kanakaraj et al. 1995). However the expression of both these proteins was unaffected after treatment with \(\beta\)GBP (Figure 6.4). Galectin 1 had been previously
been shown to down regulate Bcl-2 (Rabinovich, Alonso et al. 2000) but as previously explained the concentration of galectin1 used was much higher than that of βGBP but there has also been a report that βGBP (at a concentration similar to that used here) also decreases Bcl-2 levels in T-cells (Novelli, Allione et al. 1999). However Bcl-2 was not downregulated in CML cells by βGBP.

The apoptotic process is complex and tightly regulated and involves numerous interacting proteins. One important feature is the activation of members of the caspase family of proteases. Caspase activation proceeds as a cascade of activation of precursor caspases leading to the proteolysis and cleavage of target proteins. Caspase-3 plays an important role in activating other caspases and initiating and sustaining the apoptotic process (Dubrez, Eymin et al. 1998). However, in CML cells the activation of caspase 3 is prevented through the blockage in the release of cytochrome c (Amarante-Mendes, Naekyung Kim et al. 1998). It was speculated then that induction of apoptosis in CML cells by βGBP would activate the caspase cascade through the activation of caspase-3. Upon investigation however, it was found that there was no activation of caspase-3 (Figure 6.2) in either K562 or BV173 cells. Despite the central importance of caspase-3 activation to the apoptosis process, apoptosis induction was observed in these cell lines after treatment with βGBP, so it must involve a caspase-3 independent mechanism. Caspase-3 independent apoptosis has been observed in various other cell types after treatment with other chemotherapeutic agents e.g. staurosporine (Janicke, Ng et al. 1998). It was also reported that BV173 cells after treatment with As2O3, underwent apoptosis and exhibited downregulation of p210BCR-ABL but in a caspase-3 independent manner (Puccetti, Guller et al. 2000). The apoptotic process as can be seen in Figure 1.5 can be induced through various proteins
including other members of the caspase family thereby bypassing caspase 3 so whether this occurs in βGBP treated cells required further study.

Various investigators have shown that proteosomal inhibition in CML cells induces significant levels of apoptosis through the down regulation of p210^{BCR-ABL} (Dou, McGuire et al. 1999; Soligo, Servida et al. 2001). The proteasome/ubiquitin system controls orderly cell cycle progression through degradation of cyclins and cyclin dependent kinase (Soligo, Servida et al. 2001), therefore since βGBP inhibits cell cycle progression, was βGBP a proteosomal inhibitor, thereby inducing p210^{BCR-ABL} down regulation and apoptosis? Upon investigation, it was found that this was not the case (Figure 6.3). BV173 and K562 exhibited the down regulation of βGBP that was expected but there was no increase in the level of ubiquitin labelled proteins, which would indicate a proteosomal inhibitor. Thus p210^{BCR-ABL} down regulation was solely occurring through an inhibition of translation associated proteins namely S6 ribosomal protein as seen above.

A further investigation of other effects of βGBP treatment on CML cells focused on signalling pathways associated with p210^{BCR-ABL}. The pathways investigated were MAPK/ERK, SAPK/JNK and p38. ERK signalling is associated with mitogenic stimuli from receptors and activation of this pathway has been shown to be required for survival signalling (Mc Gee, Campiani et al. 2002). JNK and p38 proteins are activated by cellular stresses and chemotherapeutic insults such as DNA damage; UV irradiation (Kyriakis, Banerjee et al. 1994) and the activation of JNK and p38 are associated with the induction of apoptosis. Given that the response of these pathways is so different, leading to either cell survival or apoptosis, it was necessary to examine the effect if any that βGBP had on these pathways, as this may be a site of βGBP activity, which when combined with p210^{BCR-ABL} downregulation might act to induce
apoptosis in CML cells. Members of the JNK/SAPK family of proteins are activated after treatment with chemotherapeutic agents such as etoposide, camptothecin (Seimiya, Mashima et al. 1997) and along with p38 activation results in apoptosis; therefore it was assumed that these proteins would be activated by βGBP induced apoptosis. However, Figure 6.6 illustrated that after treatment with βGBP, there was no activation of either JNK1 (p46) or JNK2 (p54) at the time points examined (36 to 60 hours) or no up regulation of active p38 (Figure 6.7). There was no up regulation of active JNK or p38 proteins at time points where apoptosis was observed i.e. 48 hours. It was not surprising however that no activation of JNK proteins was observed as there are varying reports as to the role of JNK in apoptosis in CML cells treated with various agents. There seems to be a disparity between whether JNK proteins were activated or not after treatment with various agents. For example PBOX (pyrrolo-1,5-benzoxazepines) induced apoptosis requires JNK activation (Mc Gee, Campiani et al. 2002), whilst other reports have shown that induction of apoptosis in CML cells using other agents such as PD98059 does not involve any activation of JNK proteins (Kang, Yoo et al. 2000). βGBP induced apoptosis therefore did not involve the JNK pathway. The increase in JNK2 (p54) and the loss of p38 at 48 and 60 hours were judged to be not significant as both these events occurred after 48 hours. At this time point, significant levels of apoptosis were present and p210^{BCR-ABL} had already been downregulated (from 40–44 hours), and these were the phenotypes of most interest. An analysis of the effects of βGBP on ERK1/2 activation (Figure 6.5) revealed however that from 36 hours after treatment with βGBP there was a down regulation in the levels of active (phosphorylated) ERK1 (p42). As previously stated ERK proteins are associated with survival signalling. In CML cells, some of the proteins that associate with the autophosphorylated p210^{BCR-ABL}, especially Tyr177,
include Grb-2 and SOS1 (Pendergast, Quilliam et al. 1993). These proteins stabilise Ras proteins in the active state and therefore allow unregulated signalling through Ras associated proteins. Ras in its active form plays a pivotal role in many survival pathways especially MAPK associated pathways. Constitutive activation of Ras in CML cells through Grb2 and SOS1 and subsequent signalling through MAPK proteins such as ERK1/2 play an important role in the transformation of CML cells (Sawyers, McLaughlin et al. 1995). Indeed it has also been reported that Ras signalling through ERK1/2 can lead to an upregulation of ERK associated gene transcription (Kang, Yoo et al. 2000). Therefore, it could be speculated that by inhibiting the activation of ERK1, βGBP prevents the activation of one of the major signalling pathways in CML cells and especially one of the major survival signalling pathways. This perturbation in signalling in itself may be sufficient to induce the levels of apoptosis observed after βGBP treatment. This scenario was confirmed in other studies where it was found that the inhibition of ERK proteins was sufficient to induce significant levels of apoptosis in K562 cells (Kang, Yoo et al. 2000). However, there was one other interesting piece of evidence that could link p210 BCR-ABL down regulation with ERK1 activation. An examination of the pathways involved in translation (Figure 6.1) revealed that active ERK proteins promoted the activation of p70 S6 kinase, which activates S6 ribosomal protein. This was a very important observation, which could explain the reason why βGBP has such an effect on CML cells and p210 BCR-ABL expression.

Thus the following model could be proposed. βGBP inhibits the activation of Ras protein (personal communication with Livio Mallucci) through the inhibition of the conversion of RasGDP (inactive) into RasGTP (active). This would prevent the transmission of extracellular signals through the cell and prevent the activation of
downstream molecules such as ERK1 (Figure 6.5). As previously stated, a function of ERK is the activation of p70 S6 kinase, which activates S6 ribosomal protein. A loss of active S6 ribosomal protein was also observed (Figure 6.8). By down regulating S6 ribosomal protein, the translation of proteins with 5' oligopyrimidine tracts is inhibited e.g. p210BCR-ABL and this was revealed here the by a decrease in the level of p210BCR-ABL protein (Chapter 5). Thus, the main obstacle to the induction of apoptosis is removed through the inhibition of its translation and this allowed the induction of apoptosis, in the significant levels observed here (Chapter 3). This was a logical explanation linking the activity of βGBP on MAPK proteins to the down regulation of p210BCR-ABL. A model outlining the proposed scenario is illustrated below.
Proposed mechanism of action of βGBP in CML cells

As seen in Figure 1.5, the signalling pathways stimulated by expression of p210BCR-ABL are extremely complicated but both inhibiting the activity of some of the proteins involved in survival signalling and also downregulating the level of p210BCR-ABL protein, βGBP has effects on two critical components of anti-apoptotic signalling in
CML cells. Thus apoptosis is induced in a selective manner. Therefore by blocking cell signalling in cells, which have a continuous stimulus, and by directly targeting an oncogene, apoptosis would only be induced in cells exhibiting both of these criteria. The net result is that βGBP has a selective effect on CML cells and down regulates p210^{BCR-ABL} through a block in the translation mediated through an inhibition of the activation of ERK proteins and the MAPK cascade.

The most important feature of βGBP is that it is a naturally occurring molecule with cytokine properties and plays a role in the silencing phase of immune reactions (Blaser, Kaufmann et al. 1998). This offers an opportunity to utilise the activity of a naturally occurring cytokine against apoptotic resistant cells in a manner, which doesn’t have any adverse effects on normal cells. The proposed role of βGBP in an immune reaction may have an important caveat in the use of βGBP in the treatment of CML. CML patients who after conventional chemotherapy have a depressed immune system could be adversely affected by the use of another immune system suppressant. Much more study is required on the action of βGBP but it may also offer the opportunity to combine conventional treatments with these novel treatments (both at sub-lethal levels) to bring about a more favourable outcome with a less lethal effect on normal immune cells.
**Conclusion and Future Work:**

In this study, the initial aim was to examine the effect of βGBP on CML cells (both primary and cell lines). Through the use of primary cells isolated from CML patients and normal BMT donors with consent, it was established that there was a statistically significant difference between the effects of βGBP on CML derived cells versus the normal haematopoietic cells. Further study using CML cell lines determined that βGBP both induced growth inhibition and cell cycle arrest in these cell lines. These observations echoed previous work on βGBP, which determined the effect of βGBP on the cell cycle of treated cell i.e. induction of cell cycle arrest at the S/G2 transition. Despite the resistance of CML cells to apoptosis, treatment with βGBP produced significant levels of apoptosis. Upon investigation, it was discovered that the levels of p210^{BCR-ABL} protein decreased after 40–44 hours after treatment with βGBP. Therefore, βGBP treatment of CML cells was sufficient to down regulate the anti-apoptotic protein p210^{BCR-ABL}. This down regulation was found not to be through any inhibition of the transcription of p210^{BCR-ABL} mRNA. An investigation of the effects of βGBP on survival signalling and apoptotic pathways revealed interesting results. There was no down regulation of anti-apoptotic proteins such as Bcl-XL or Bcl-2. Also, there was no up or down regulation of the activity of signalling pathways such as JNK1/2 or p38. However, there was an observed down regulation in the activity of ERK1 and also upon investigation of proteins downstream of MAPK associated proteins revealed that the activity of the translational protein S6 ribosomal protein was also down regulated. In summary, βGBP has been reported to inhibit the activity of Ras and it was found in this study here that a protein down stream from Ras, ERK1 was also inhibited. This resulted in the down regulation in the activity of S6 ribosomal protein, which was necessary for the translation of proteins with oligopyrimidine
tracts (of which p210^{BCR-ABL} was one). This brought about the downregulation of translation of p210^{BCR-ABL} protein. A drop in the level of p210^{BCR-ABL} removed the block in apoptosis in CML cells and this combined with multiple aberrations in signalling and the induced cell cycle block contributed to the induction of apoptosis.

The importance of these results is that, βGBP is a naturally occurring cytokine with an immunological function (Blaser, Kaufmann et al. 1998). This study has indicated that this negative regulator of the cell cycle exhibits anti-leukaemic activity. The results revealed here add to the previously known observations of βGBP as a selective anti-cancer agent. A larger cohort of patient samples (both CML and normal) is required to fully assess the selective effect of βGBP and also patients at different stages of the disease (blast crisis and accelerated phase) but the results here suggest that βGBP may have an important role to play as a potential purging agent particularly in the context of minimal residual disease.

Further study is also required to evaluate the downstream effects of βGBP on CML cells. The specificity of βGBP relates to its effects on p210^{BCR-ABL} downstream signalling. Therefore by further discerning the pathway and proteins affected by βGBP, it may be possible to that this protein may have anti-cancer activity against a wider range of cancers apart from leukaemia.
Appendices
Appendix 1:

1.1 Examination of cell lines for *Mycoplasma* contamination

It has been known for many years that *Mycoplasma* and related *Acholeplasma* species are serious, widespread and persistent contaminants of cell cultures. It is one of the commonest forms of contamination of cell lines and it has been regularly shown that up to 60% of cell cultures are contaminated with Mycoplasma (McGarrity, Vanaman et al. 1984). The presence of Mycoplasma is not toxic or does not destroy host cells but can have other consequences such as changes to cell metabolism, altered DNA/RNA synthesis and altered growth and morphology. Therefore the presence of Mycoplasma can lead to false and unreliable results. Therefore it extremely important to regularly test for contamination by Mycoplasma and this is now a pre-requisite for publication in many journals.

There are various techniques used for the detection of Mycoplasma e.g. DNA staining with dyes such as DAPI or bisbenzimide. However the technique employed here was a PCR based assay, which relies on the amplification of a Mycoplasma specific DNA sequence followed by an ELISA based detection of the products of this PCR reaction. The sensitivity of this kind of assay was $1 \times 10^3$ colony forming units per millimetre of culture medium.

The assay was carried out as follows (as per kit protocol, Roche). All solutions unless otherwise stated were part of assay kit, Mycoplasma PCR/ELISA™. 1ml of cell culture media was centrifuged at 1500rpm to pellet the cells and the resulting supernatant was then centrifuged at 12,400rpm for 10 minutes to sediment any Mycoplasma present. Following removal of this supernatant, 10μl of UV treated water and 10μl lysis buffer was added. A positive control containing 10μl positive control DNA plus 10μl lysis solution was set up as was a negative control sample...
containing 10μl UV treated water plus 10μl lysis solution. These solutions were incubated for 1 hour at 37°C followed by addition of 30μl neutralisation solution. 10μl of each sample was added to 10μl of the PCR mix plus 15μl sterile water. The PCR reaction was carried out as follows: 5 min at 95°C, 40 cycles of 94°C for 30 secs, 62°C for 30 secs, 72°C for 1 min and then 72°C for 10 minutes. 40μl of denaturation reagent was added to 10μl of PCR product and incubated for 10 minutes at room temperature. To these reactions, 450μl of hybridisation reagent (445μl hybridisation buffer per reaction plus 4.45μl biotinylated probe per reaction) was added and 200μl of this solution was transferred to well of ELISA plate. The plate was covered with protective foil and incubated at 37°C for 3 hours shaking at 150rmps. After this time period, the solution was removed and the wells washed three times with washing buffer (10X washing buffer 0.2ml per reaction plus 1.8ml sterile water per reaction). 200μl anti-DIG POD (2μl anti-DIG POD per reaction plus 198μl conjugate dilution buffer per reaction) was added to each well and incubated at room temperature for 30 minutes, shaking at 150rmps. After incubation, wells were again washed three times with washing buffer. The TMB substrate was warmed to room temperature and 100μl was added to each well. This reaction was incubated at room temperature for 20 minutes, shaking at 150rmps, after which 100μl stop reagent was added.

The A690 and A450 were measured using a spectrophotometer (Tecan) and the A450 – A690 was calculated for each sample. Negative control samples should be less than 0.25 and positive samples should be higher than 1.2. The (A450 – A690 of sample) – (A450 – A690 for negative control) was calculated and a positive result for Mycoplasma is regarded as any sample with a value of 0.2 or higher.
Appendix 2:

2.1 Expression of Galectin 1 in Chronic Myeloid Leukaemia cell lines:

As previously outlined, lectins are carbohydrate binding proteins, which share the ability to bind β-galactosides through conserved carbohydrate recognition domains (Barondes, Cooper et al. 1994). There are 14 described members of the Galectin super family but four well described members. For the purposes of this thesis, it was Galectin-1 that was of most interest. The properties of Galectin-1, outlined previously include cell adhesion, cell differentiation and growth and immune cell regulation. Galectin1 can exist as either a homodimers or as a monomer (14kD). It was this monomeric form (βGBP) that was the subject of this thesis. Both proteins are the product of the same gene, namely LGALS1. However, there are differences between the modes of action of the monomeric form versus the dimeric form, with the monomer βGBP functioning as a cytokine as opposed to the lectin properties of Galectin-1. What was described in this thesis was the action of βGBP on CML cells, both primary material and cell lines. The response of CML cells to βGBP was found to be the induction of significant levels of apoptosis and a selective effect between normal and neoplastic cells (Chapter 4). Therefore, it was decided to examine whether there was expression of Galectin-1 at both the mRNA level and protein level in CML cell lines. The reason this was examined was that for one, Gal-1 is expressed from chromosome 22, the same chromosome affected in CML as the Philadelphia chromosome. Therefore it may be the case that the expression of Gal-1 may be affected in the modified chromosome 22. If Gal-1 acts as a cancer surveillance molecule, reduced expression in cancer cells when compared to normal cells may provide evidence for this hypothesis.
Results:

A 2.1 Expression of Galectin 1 mRNA expression:
In order to qualify whether there was expression of Galectin-1 mRNA, quantitative PCR TaqMan technology (as described in Chapter 5) was employed. Forward and reverse primers and a TaqMan probe were designed for the Galectin-1 gene (Materials/Methods Table 2.4) using Primer Express™ (Applied Biosystems).

A 2.1.1 Galectin 1 primer and probe optimisation for Galectin 1 mRNA:
The first step in any experiment employing TaqMan technology is the optimisation of the primers and probes involved in the PCR reaction in order to minimise the concentrations needed to achieve the maximum R_m. Primer and probe optimisation curves were set up as outlined in Materials/Methods 2.6.5. The amplification plots and the C_T values obtained in the primer optimisation plots are outlined in Figure A2.1. An analysis of the threshold values (C_T's) indicated that there was very little difference between the C_T values of the various forward: reverse primer combinations. Therefore the combination of 100nM for both forward and reverse primer for Galectin-1 was selected for use. For the probe optimisation curves, reactions were carried out as in Materials/Methods 2.6.4 and 2.6.5. Figures A2.2 illustrate the amplification plots and C_T values for the probe optimisation experiments for Galectin-1. From these C_T values, an optimum concentration of 150nM of probe was chosen.

Primer and probe concentrations for the internal control gene GAPDH were identical to those determined in Chapter 5 Figure 5.7 and 5.8 i.e. 150nM for both forward and reverse primer and a probe concentration of 100nM.
A2.1.2 Examination of the expression of Galectin 1 in CML cell lines:
The expression of Galectin-1 was determined using identical TaqMan reaction conditions as those used in Materials/Methods 2.6.4. cDNA was made from RNA extracted as previously described (Materials/Methods 2.6.1 and 2.6.3) from CML cell lines (BV173, K562, LAMA84 and KYO-1) and also non-CML cell lines (Jurkat E6.1 and HL-60). cDNA was also made from RNA extracted from a normal donor cell sample. Each sample was then analysed for both Galectin-1 RNA expression and also GAPDH expression to act as an internal control. It was evident that each cell line expressed Galectin-1 with CT values ranging from 18 to 26. The triplicate CT values obtained for the expression of each gene examined are outlined in Figure A2.3a. The variance that was evident in the CT values for Galectin-1 expression was also evident in the GAPDH values for each cell type with values ranging from 15 to 20. An example of the amplification plots for both Galectin-1 and GAPDH are illustrated in Figure A2.3b.

A2.1.3 Analysis of expression of Galectin-1:
The variance in the expression for both Galectin-1 and GAPDH meant that it was difficult to relate the expression of each gene in a cell line to another cell line. Therefore in order to fully quantify the expression of Galectin-1, it was necessary to employ the comparative CT method (Materials/Methods 2.6.7). The triplicate mean values were used to calculate ΔCT (CT Galectin-1 – CT GAPDH) for each cell type. In order to calculate ΔΔCT for each cell type, the ΔCT for the normal sample was subtracted from each other ΔCT value. The normal ΔΔCT value was again used to calculate the index value where it was assigned as the calibrator figure. This meant that each cell type was referenced back to the expression of Galectin-1 in the normal.
cell sample and the results are outlined in Figure A2.4. Each of the CML and non-CML cell lines have expression indexes, which are well below the index value of 1 for normal cells meaning that all of the cell lines examined have a lower level of expression than that seen with normal cells.

**A2.2 Expression of Galectin 1 protein in CML cell lines:**
To investigate whether there was Galectin-1 expression in CML cell lines, protein was extracted from each of the selected cell lines, K562, BV173, LAMA 84 and KY-O1 and western blotting as previously outlined was carried out using an anti-GAL-1 antibody. A T-cell leukaemia cell line Jurkat E6.1 was also used along with a promyelocytic leukaemia cell line HL-60. DU145, a prostate cancer cell line was used as a positive control for Galectin-1 expression. To act as a normal control, protein was extracted from a normal cell sample. Probing for α-actin controlled for equal loading of protein. Figure A2.5 indicates that the highest expression of Gal-1 was in the CML cell line KYO-1, with a lower level being expressed by the CML lines K562 and LAMA84. There was no detectable expression of Gal-1 in BV173 or the non-CML cell lines HL60 and Jurkat E6.1.
Discussion:

The main aim of this part of this thesis was to establish whether if the molecule protein Galectin-1 was expressed in the CML cell lines used in the main body of this thesis. As previously explained, Galectin-1 is the dimeric form of β-Galactoside binding protein, which was the molecule under study in this thesis. However, there was no available antibodies that would allow for a Western blot for the monomeric form of Galectin-1, namely β-Galactoside binding protein. It was therefore decided to look at the expression of the dimeric form, namely galectin1 and this would give an indication of the level of expression of this Galectin family member (both dimeric and monomeric forms). A previous report had shown that there was a very low expression of Gal-1 in K562 cells compared to other examined CML cell lines (Lutomski, Fouillit et al. 1997). This was speculated to be due to the presence of the gene for Galectin-1 on chromosome 22; the chromosome involved in the translocation event in CML and this may interfere with the expression of Galectin-1. An identical situation was observed here with a different level of expression observed in each cell line examined Figure A2.5, however the K562 cells examined expressed quite a high level of Gal1 compared to the positive control cells DU145. The discrepancy between the results here and those of the previous study may be due to the antibodies used or the means of detection, Western blotting here and 2D-PAGE/Western blotting in (Lutomski, Fouillit et al. 1997).

However upon examination of RNA expression, each of the cell lines expressed RNA for Galectin-1 (Figure A2.3). An analysis of the levels of expression by the comparative \( C_T \) method was used to equalise each cell line against one another due to the different levels of expression of GAPDH, the internal control gene. The normal cell sample was used as the calibrator against which all the other samples were
compared. It was evident that each of the cell lines, which were all cancer cell lines, had a lower level of expression compared to the normal cells (Figure A2.4). The index values compared favourably with (Figure A2.5) and protein expression in the cell lines, with the cell lines, which express the higher levels of RNA having a detectable level of Gal-1 protein whilst those with a low index value not having a detectable level of protein expression. This may be due to the antibody having a threshold below which it won’t detect Gal-1.

The most important result to take from this is the difference in expression between normal cells and cancer cells. A previous property of Galectin-1 was found to be in the silencing phase of an immune reaction (Blaser, Kaufmann et al. 1998) but also had a role to play in cell cycle regulation (Wells and Mallucci 1991). However it has been shown in this thesis and in previous work (Wells, Davies et al. 1999) that βGBP had a role to play in the control of cancer cell growth and could induce apoptosis in cancer cells whilst leaving normal cells unaffected. This “immune surveillance” role has been speculated to be another role of βGBP and Galectin-1 and may explain why the cancer cell lines above express such a low level of Gal-1 protein and allows cancer cells to evade apoptosis.

Further investigation of the expression of Gal-1 in cancer cells is required to fully examine whether there is a differential level of expression between normal and cancer cells. In the context of this study, an examination of the level of expression in normal cells versus CML cells isolated from CML patients would reveal the importance of the expression of Galectin-1 in this disease.
Figures:

(a) Amplification - bGBP primer curve

(b) Primer Concentration (nM) | Galectin-1 C<sub>T</sub> Values (average) | Colour Reference
--- | --- | ---
100/100 | 27.86 | ■
100/300 | 27.50 | ■
300/900 | 26.69 | ■
300/100 | 27.91 | ■
300/300 | 27.23 | ■
300/900 | 27.13 | □
900/100 | 28.33 | ■
900/300 | 27.31 | ■
900/900 | 27.04 | ■

Figure A2.1: Primer optimisation curve for Galectin 1 primers (forward and reverse)

TaqMan reactions were set up as outlined in Materials/Methods 2.6.4 and primer (forward and reverse) concentrations were varied as in Materials/Methods 2.6.5. (a) illustrates amplification curves for each forward/reverse primer concentration. (b) outlines mean Galectin-1 C<sub>T</sub> values for each primer set concentration.
Figure A2.2: Probe optimisation curve for Galectin 1 probe

TaqMan reactions were set up as outlined in Materials/Methods 2.6.4 and probe concentration varied while primer concentrations were kept constant. (a) illustrates amplification curves for each probe concentration. (b) outlines mean Galectin-1 $C_T$ values for each probe concentration.

<table>
<thead>
<tr>
<th>Probe Concentration (nM)</th>
<th>Galectin-1 $C_T$ Values (average)</th>
<th>Colour Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>28.98</td>
<td>■</td>
</tr>
<tr>
<td>100</td>
<td>27.95</td>
<td>■</td>
</tr>
<tr>
<td>150</td>
<td>27.80</td>
<td>■</td>
</tr>
<tr>
<td>200</td>
<td>27.91</td>
<td>■</td>
</tr>
<tr>
<td>250</td>
<td>27.84</td>
<td>■</td>
</tr>
</tbody>
</table>
Appendices

(a) [Graph showing amplification in cell line gbp 1]

(b) [Graph showing amplification in cell line gap no2]

(c) | Cell Line | Galectin 1 C<sub>T</sub> Values (average) | GAPDH C<sub>T</sub> Values (average) |
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>K562</td>
<td>17.931 18.105 18.042</td>
<td>15.780 15.627 15.689</td>
</tr>
<tr>
<td>BV173</td>
<td>22.839 22.578 22.938</td>
<td>17.365 17.695 18.036</td>
</tr>
<tr>
<td>LAMA84</td>
<td>18.157 18.218 18.062</td>
<td>15.459 15.984 15.828</td>
</tr>
</tbody>
</table>
Figure A2.3: Galectin-1 expression in cell lines
TaqMan reactions were set up as outlined in Materials/Methods 2.6.4 and probe and primer concentrations used for both Galectin-1 and GAPDH were as determined in A2.1, A2.2 and Chapter 5 (a) and (b) illustrates amplification curves for each Galectin-1 and GAPDH respectively. (c) outlines mean Galectin-1 and GAPDH C_T values for cell line.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>ΔC_T (galectin 1 – GAPDH)</th>
<th>ΔΔC_T (ΔC_T - ΔC_T normal)</th>
<th>INDEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>2.337</td>
<td>2.534</td>
<td>0.172</td>
</tr>
<tr>
<td>BV173</td>
<td>4.749</td>
<td>4.946</td>
<td>0.032</td>
</tr>
<tr>
<td>LAMA84</td>
<td>2.317</td>
<td>2.514</td>
<td>0.175</td>
</tr>
<tr>
<td>KY-O1</td>
<td>3.131</td>
<td>3.328</td>
<td>0.099</td>
</tr>
<tr>
<td>Jurkat E6.1</td>
<td>9.406</td>
<td>9.603</td>
<td>0.001</td>
</tr>
<tr>
<td>HL60</td>
<td>1.784</td>
<td>1.981</td>
<td>0.250</td>
</tr>
<tr>
<td>Normal</td>
<td>-0.197</td>
<td>0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Figure A2.4: Analysis of Galectin-1 expression in cell lines
Comparative C_T method from Materials and Methods 2.6.7 was used to calculate relative level of expression of Galectin-1 using normal cell as calibrator. Index refers to 2^ΔΔC_T, which relates amount of target to calibrator.
Figure A2.5: Western blot of Galectin 1 expression in cell lines.
Protein lysates were prepared as in Materials and Methods 2.5.2.1 and probed for the expression of Galectin 1 and actin as a control for equal loading. Lane (1) DU145 positive control, (2) K562, (3) BV173, (4) LAMA 84, (5) KY-O1, (6) HL-60, (7) Jurkat E6.1.
Bibliography


