In the invention, a polypeptide comprising at least six contiguous amino acid residues of the amino acid sequence of one or more of (i) HGPLRD WCLFG, or (ii) GGD YCCLRYV, or (iii) WGGVMVLTWP, or (iv) VRGYGASLLGLPISAV, or (v) an amino acid sequence having at least 70% identity to the sequence of any one of (i) to (iv) across the full length of said sequence. The invention also relates to uses of the peptide in gene expression, in BCL-6 inhibition and in medicine.
Field of the Invention

The invention relates to inhibitors of BCL-6. In particular, the invention relates to small peptide inhibitors of BCL-6 such as aptamer 48 disclosed herein.

Background to the Invention

BCL-6 is a transcription factor essential for germinal centre B cell development. BCL-6 is a transcriptional repressor whose N-terminal POZ-domain mediates protein-protein interactions to assert its effects.

BCL-6 is involved in diffuse large cell lymphoma and is over expressed in other types of non Hodgkin's lymphoma and in high grade breast cancer. Furthermore, BCL-6 is expressed in other lymphoproliferative disorders such as follicular lymphoma, Burkitt's lymphoma and B-cell predominant Hodgkin's disease.

Furthermore, knockout mouse studies show that deletions in the BCL-6 reveal an essential role in germinal centre formation and the mediation of humoral immunity.

No repressor of BCL6 is known in the art. The only example of a molecule capable of interfering with BCL6 activity known in the art is disclosed in Polo et at (2004 Nature Medicine Volume 10 pages 1329-1335). Polo et al disclose a potential repressor derived from SMRT (silencing mediator of retinoid and thyroid receptors).

The only clinically available therapy directed at B-cells consists of the anti CD20 antibody. This antibody can be used to eliminate B-cells. However, this has the drawback that it is non-selective. For example, BCL6 over expressing B-cells cannot be selectively destroyed by the anti CD20 antibody. Thus, in a patient subjected to anti CD20 antibody therapy, massive global loss of B-cell occurs which results in immune compromise and related problems.
An alternative therapy which has been proposed in this field is a targeting of histone deacetylases (HDAC). These are recruited by BCL6 in order to turn promoters off. Inhibitors of this activity (HDACi) turn the promoters back on i.e. their promoters remain acetylated due to inhibition of the deacetylases. However, as will be appreciated this is a highly non-specific approach. Wholesale inhibition of histone deacetylases is itself problematic. Furthermore, the effects of such a treatment can be far reaching and are ill characterised.

Thus, there is a need for novel repressors of BCL-6 activity.

The present invention seeks to overcome problems associated with the prior art.

Summary of the Invention

The present inventors have undertaken a large scale screen for interactors of BCL-6. They have surprisingly discovered a number of BCL-6 repressing peptides. These peptides are disclosed herein in the form of peptide aptamers, having core unique sequences of approximately 10 amino acids.

As well as having a clear protein-protein interaction with BCL-6, the peptides disclosed herein have been shown to be functionally capable of repressing BCL6 activity. Furthermore, as a result of these studies, a specific novel surface has been delineated on BCL-6 through which repressor activity can be mediated.

The present invention is based on these surprising findings.

Thus, in one aspect the invention provides a polypeptide comprising at least six contiguous amino acid residues of the amino acid sequence of one or more of

(i) HGPRDWCLFG, or

(ii) GGDYCLRYV, or
(iii) WGGVMLVTWP, or
(iv) VRGYGASLLLGPPISAV, or
(v) an amino acid sequence having at least 70% identity to the sequence of any one of (i) to (iv) across the full length of said sequence.

In another aspect, the invention provides a polypeptide as described above wherein said peptide comprises the full amino acid sequence of one or more of

(i) HGPRDWCLFG, or
(ii) GGDYCCLRYY, or
(iii) WGGVMLVTWP, or
(iv) VRGYGASLLLGPPISAV.

In another aspect, the invention provides a polypeptide as described above wherein said sequence is HGPRDWCLFG (Aptamer 48).

In another aspect, the invention provides use of a polypeptide as described above in the expression of a BCL6 repressed gene.

In another aspect, the invention provides use of a polypeptide as described above in the inhibition of BCL6.

In another aspect, the invention provides a use of a polypeptide as described above in the blocking of the POZ site of BCL6.

In another aspect, the invention provides a method of inducing expression of a gene selected from the group consisting of CD69, blimp 1, and cyclin D2 in a cell by contacting said cell with a polypeptide as described above.

In another aspect, the invention provides a method as described above wherein the cell is a cell of the B cell lineage.
In another aspect, the invention provides a method of inhibiting the effects of BCL6 overexpression comprising contacting a cell overexpressing BCL6 with a polypeptide as described above.

In another aspect, the invention provides a method of inducing differentiation of a proliferating B cell comprising contacting said B cell with a polypeptide as described above.

In another aspect, the invention provides a polypeptide as described above for use as a medicament.

In another aspect, the invention provides a polypeptide as described above for use in the treatment of a lymphoproliferative disorder.

In another aspect, the invention provides use of a polypeptide as described above in the manufacture of a medicament for the prevention or treatment of a lymphoproliferative disorder.

In another aspect, the invention provides a method for causing growth arrest of a cell overexpressing BCL6 comprising contacting said cell with a polypeptide as described above and at least one cytokine. In another aspect, the invention provides a composition comprising a polypeptide as described above and at least one cytokine; preferably the polypeptide is or comprises Aptamer48. Preferably said composition is for use in medicine; preferably said composition is for use in the manufacture of a medicament for the prevention or treatment of a lymphoproliferative disorder.

In another aspect, the invention provides a polypeptide comprising an amino acid sequence selected from the group consisting of Aptamer 7, Aptamer 48, Aptamer 52 and Aptamer 58.

In another aspect, the invention provides a polypeptide consisting of DILTDVVIVVSREQFRAH.
In another aspect, the invention provides a polypeptide comprising DILTDVVIVVSREQFRAH for use as a medicament.

In another aspect, the invention provides a polypeptide comprising DILTDVVIVVSREQFRAH for use in the treatment of a lymphoproliferative disorder.

Preferably the peptide comprising DILTDVVIVVSREQFRAH consists of DILTDVVIVVSREQFRAH.

In another aspect, the invention provides use of a polypeptide comprising DILTDVVIVVSREQFRAH in the manufacture of a medicament for the prevention or treatment of a lymphoproliferative disorder.

The invention relates to peptide aptamers to antagonise BCL-6 function.

The peptides of the invention find application in disorders such as B Cell lymphoma.

Preferred lymphoproliferative disorders are follicular lymphoma, Burkitt's lymphoma and B-cell predominant Hodgkin's disease

The invention also relates to uses of the peptides in gene expression, in BCL-6 inhibition and in medicine.

Detailed Description of the Invention

For some aspects of the invention, the closest prior art may be considered to be Polo et al (2004 Nature Medicine pages 1329-1335). However, the peptides of the present invention are dramatically different from those disclosed in Polo et al. Comparative data establishing this is presented in the examples section. Firstly, the peptide disclosed by Polo et al is divergent in sequence from the peptides disclosed herein. Secondly, there is only a weak homology between the sequences disclosed herein and
those of Polo et al. Thirdly, with reference to Figure 2b, clear functional differences are shown between the prior art peptide (BBD) and an exemplary peptide of the present invention (aptamer 48). Fourth, with reference to the table in Figure 2c, further differences are illustrated, such as the domain on BCL6 with which the peptides interact. In particular, rows 1 and 2 of the table of Figure 2c illustrate precisely opposite binding patterns between peptides of the prior art (BBD) and of the invention (for example aptamer 48). Fifth, in order to explore more fully the differences between the invention and the prior art, aptamer 48 was mutated to be more like the peptide of the prior art. This resulted in loss of interactive activity of the hybrid peptide. Sixth, the prior art peptide and the peptides of the present invention have been shown to bind different structural elements of the BCL6 protein.

Overview

BCL-6 is a transcription factor essential for germinal centre B-cell development. The BCL-6 gene is involved in diffuse large cell lymphoma and over-expressed in other types of non-Hodgkin's lymphoma and in high grade breast cancer. BCL-6 is a transcriptional repressor whose N-terminal POZ domain mediates protein-protein interactions to exert its effects. Reasoning that disruption of POZ domain-mediated interactions may be an effective route to antagonizing the effects of BCL-6 in lymphoma, we screened a library for peptide aptamers that specifically bind to BCL-6 POZ and not the POZ domains of related proteins and describe here the first set of these reagents, most preferred example of which is Apt48. Apt48 binds BCL-6 POZ in a manner distinct from the transcriptional co-repressor SMRT, yet was found to prevent BCL-6 mediated repression of a luciferase reporter gene. Apt48 also reproduced several previously validated effects of BCL-6 inhibition. Notably, expression of the differentiation markers CD69, Blimp-1 and cyclin D2 was increased in B-cell lines when Apt48 was expressed. We also show that expression of Apt48 restores cytokine-mediated growth arrest to BCL-6 over-expressing cells. Thus, we have identified a peptide aptamer that affects a function of BCL-6 that is required to prevent differentiation of proliferating B cells.
BCL-6

BCL-6 is a transcription factor which is normally expressed at high level in lymph node germinal centres (Cattoretti et al., 1995; Onizuka et al., 1995). The BCL-6 gene was cloned from chromosomal translocations in diffuse large cell lymphoma (Baron et al., 1993; Ye et al., 1993), suggesting that it has a role in the causation of this disease, and is expressed in other lymphoproliferations e.g. follicular lymphoma, Burkitt's lymphoma and B-cell predominant Hodgkin's disease and a number of breast cancers (Logararjah et al., 2003).

Analysis of mice bearing homozygous deletions of the BCL-6 locus demonstrates an essential role in germinal centre formation and humoral immunity (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). BCL-6 expression promotes proliferation and inhibits differentiation and apoptosis of B-cell lines (Niu et al., 1998; Shaffer et al., 2000). Conversely, expression of an inducible dominant negative BCL-6 (Shaffer et al., 2000) led to up-regulation of genes associated with B cell differentiation e.g. Blimp-1, and cell death in the Burkitt's lymphoma cell line Raji (Turner et al., 1994).

Similar results were obtained in another Burkitt's lymphoma cell line where cross-linking of surface IgM led to down regulation of BCL-6 and apoptosis (Niu et al., 1998). Thus BCL-6 allows proliferation and its down regulation is associated with differentiation and cell death. These data suggest that BCL-6 may be a therapeutic target in lymphoma.

At the molecular level, the carboxy-terminus of BCL-6 comprises a DNA binding domain of 6 zinc-fingers, which recognise the same consensus sequence as that of the signal transducers and activators of transcription (STAT) family of transcription factors (Dent et al., 1997). BCL-6 can bind to STAT6 (Dent et al., 1997, Harris et al., 1999) and STAT3 (Reljic et al., 2000) binding sites, suggesting that it may function in part by antagonizing STAT signalling. The effector portion of BCL-6 is a POZ/BTB domain (Pox virus and zinc finger/bric-a-brac tramtrack broad complex (Bardwell and Treisman, 1994); hereafter referred to as the POZ domain) located at the amino terminus of the molecule. POZ domains mediate transcriptional repression (Chang et al., 1996) and this is generally, although not exclusively (Deltour et al., 1999), achieved by association with the co-repressor proteins N-CoR and SMRT (Huynh et al., 1998), which in turn recruit histone deacetylases (Dhordain et al., 1998). As well as these
heterologous protein-protein interactions, POZ domains are able to homodimerise, and in some cases heterodimerise (Davies et al., 1999; Dhordain et al., 2000 and references in Table 1). It has been postulated that overexpression of one POZ domain-containing protein may lead to heterodimerisation with another POZ domain-containing protein, resulting in loss of function and either physiological or pathological effects (Bardwell and Treisman, 1994). This mechanism could plausibly contribute to pathology in cells over-expressing BCL-6 as a result of chromosomal translocation.

Elucidation of the crystal structure of the homodimer formed by the POZ domain of PLZF (promyelocytic leukaemia zinc finger; Ahmad et al., 1998; Melnick et al., 2000) has demonstrated that there is a large interface at which the components of the homodimer interact. A relatively small charged pocket, formed from both halves of the dimer, is required for recruitment of co-repressor molecules and is hence essential for POZ domain function. A more recent structure of the BCL-6 POZ domain shows that a peptide from each of two SMRT proteins bind symmetrically to the BCL-6 POZ homodimer (Ahmad et al., 2003). Structural information was used to design a SMRT peptide derived inhibitor of BCL-6 function, called BBD (BTB Binding Domain) (Polo et al., 2004).

Here, we describe the isolation of peptide aptamers (peptides whose amino- and carboxy-termini are anchored in the context of a scaffold protein; Colas et al., 1996) against BCL-6. The role of the scaffold protein is first, to reduce the range of conformations that can be adopted by a given sequence of amino acids; second, to display these conformationally-constrained peptides on the surface of a larger molecule; and third, to stabilise the presented peptide in the cellular environment (Colas et al., 1996). As is the case in proteins that occur in nature, different peptide sequences adopt different conformations. Libraries of constrained peptides therefore represent a range of structures that can be screened for biological interactions, with a view to identifying molecules with desired effects. Constrained peptides are able to bind to their target proteins with specificities and affinities comparable to antibodies (Colas et al., 1996) and have been used to interfere with protein function in a number of cellular pathways (see Crawford et al., 2003 for references). Like the SMRT-derived peptide, by binding to the POZ domain of BCL-6 peptide aptamers may specifically compete for individual protein-protein interactions in vivo. In contrast to the SMRT-
derived peptide, peptide aptamers identified in our screen may bind to any of several protein-interaction surfaces of the BCL-6 POZ and may therefore identify other ways of interfering with BCL-6 function. We characterise the peptide aptamers we have isolated, the most preferred example of which is termed 'Apt48'. Our data show that Apt48 binds to BCL-6 POZ in a manner distinct from that used by the SMRT peptide.

Apt48 is able to interfere with BCL-6 mediated repression in three distinct cellular assays, and restores on BCL-6 over-expressing cells the ability to growth arrest in response to cytokines. Our results identify the POZ domain of BCL-6 is a valid drug target and that its function may be inhibited by targeting sites other than the groove where SMRT-related co-repressors bind.

Since its discovery as a translocation partner of the immunoglobulin heavy chain in diffuse B-cell lymphoma (Baron et al 1993; Ye et al 1997) there has been intensive study of the biology of BCL-6 and much is now known about the molecular mechanisms by which it regulates the expression of target genes (Dent et al, 1997; Shaffer et al, 2000; Harris et al, 1999). Functional studies demonstrate that BCL-6 allows cell proliferation but prevents B-cell terminal differentiation (Niu et al, 1998; Shaffer et al, 2000; Reljic et al, 2000) and this suggests a means by which its constitutive expression promotes the development or growth of lymphomas. However, it was not clear whether inhibition of BCL-6 function would be sufficient to reverse the proliferative phenotype: as a transcriptional regulator, it is possible that changes in gene expression due to altered BCL-6 function are themselves tumorigenic, and inhibiting BCL-6 may not be able to reverse the effects of these down-stream genes.

BCL-6 must homodimerise through its N-terminal POZ domain and recruit co-repressor molecules and histone deacetylases to mediate its effects (Huynh and Bardwell, 1998; Dhordain et al, 1998; Ahmad et al, 2003). We reasoned that disruption of interactions mediated by the POZ domain with small molecules was feasible and likely to result in inhibition of BCL-6 function. Knock out mouse studies in which BCL-6 expression was absent from the earliest stages of development produces impaired primary and secondary T cell-dependent immune responses and the development of a Th2 inflammatory reaction (Dent et al, 1997; Ye et al, 1997). The effects of BCL-6 deficiency induced in mature animals, are impossible to
predict and at present targeting BCL-6 appears to be a route that will provide new treatments for lymphomas.

We have taken an open-ended approach to the inhibition of BCL-6, in seeking to identify small proteins that, by binding to the POZ domain, would inhibit BCL-6 function and allow the expression of genes usually repressed by BCL-6. We concentrated on the most preferred inhibitor, termed Apt48, whose strength of interaction with BCL-6 is comparable to that of the native protein-protein interaction of the POZ domain itself in the yeast two hybrid format. We show that Apt48 is able to reverse BCL-6 mediated repression of three target genes (CD69, Blimp-1 and cyclin D2).

We have evaluated Apt48 in 6 different cell lines, that either do not express endogenous BCL-6 (U2os, BCL-I), or express pathological levels of BCL-6 (mouse A20, human Burkitt's lymphoma lines Ramos, Raji and Daudi). In U2os cells, we show that Apt48 can interact with transfected, over-expressed BCL-6. We use the cytokine-responsiveness of BCL-I cells to show that Apt48 can restore cytokine-mediated induction of gene expression at a promoter otherwise repressed by BCL-6. In ramos and A20 cells, we show that Apt48 can lead to the de-repression of three genes that are known targets of BCL-6 mediated repression. Moreover, we show that Apt48 can restore clinically-relevant growth arrest to BCL-6 over-expressing lymphoma cells.

The BBD peptide derived from the portion of the SMRT co-repressor that binds to BCL-6 has recently been shown to be able to inhibit BCL-6 function (Polo et al, 2004). It was important to find out whether the mechanism of action of Apt48 involves inhibition of SMRT binding at the site occupied by the BBD peptide. However, when we altered the amino terminal sequences of the random portion of Apt48 to more closely resemble the SMRT sequence, we found that interaction with BCL-6 POZ was lost. Conversely, a mutation in BCL-6 POZ that inhibits the recruitment of the SMRT derived peptide does not inhibit recruitment of Apt48 (Figure 2B). We mapped the interaction site of Apt48 on the BCL-6 POZ to an 18-
amino acid residue fragment that contributes two strands of beta sheet at the top of
the POZ monomer in the structure, and falls within a region surrounding the charged
pocket (Ahmad et al 2003; Figure 2C). The BBD peptide, in both the structure and in
our yeast two hybrid mapping experiment, binds at the opposite end of the molecule.

We conclude that Apt48 is binding to BCL-6 in a manner distinct from that used by
SMRT, in a region of beta sheet at the top of the molecule (Ahmad et al, 2003).
Further experiments involving site directed mutagenesis of this region in the POZ
construct both in yeast and mammalian systems will be required to verify this. The
ability of full length POZ to interact with this region (Figure 2C) is suggestive of a
topological surface capable of recognising some orientation(s) of a POZ domain.
Keeping in mind that this region is close in proximity to the charged pocket at the
top of the molecule that has previously been suggested to serve as a protein
interaction site (Melnick et al, 2002), it is quite likely that the region may be part of
a binding site of yet uncharacterised interaction partners, perhaps containing POZ
domains themselves. The ability of Apt48 to bind to this region may represent a novel
mechanism of action where it sterically competes or displaces native interactors
required for proper functioning of BCL6. Thus, our open-ended approach has led us
to identify a new way to interfere with BCL-6 function, distinct from that used by

The mechanism of action of Apt48 may advantageously be further studied using the
reagents and techniques disclosed herein. It is a possibility that Apt48 may compete
for binding with a region of SMRT (or another co-repressor) that makes contact at
the top of the molecule. However, although such an interaction has been implied
(Melnick et al, 2002), experimental proof for such an interaction is lacking (Ahmad
et al, 2003). It is a formal possibility that Apt48 may inhibit DNA binding by full
length BCL-6. However, POZ domains have been suggested to inhibit DNA binding
by the carboxy-terminal zinc finger domain (Bardwell and Treisman, 1994), so that it
might be anticipated that Apt48 will increase sequence-specific DNA binding by the
zinc finger domain.
The co-repressors N-CoR and SMRT recruit histone deacetylase in order to accomplish transcriptional repression (Catoretti et al., 1995), but more recently other interacting repressor proteins have been identified. ETO binds to the fourth zinc finger of BCL-6 and also recruits histone deacetylase and the Mi-2/NURD co-repressor complex associates with its central region. This means of mediating transcriptional repression was shown to be functionally significant in reprogramming B-cell differentiation (Fujita et al, 2004). Thus although the efficacy of blocking the SMRT/BCL-6 interaction has been shown (Polo et al, 2004) there is therapeutic value in employing other means of abrogating BCL-6 function, such as those disclosed herein. Furthermore, the peptides of the invention find further application in the development of such agents.

Thus we have validated a novel surface of BCL-6 as a therapeutic target in vivo. Apt48 can now be used or optimized as a clinically useful agent. There are several independent strategies which may be employed to refine the peptides of the invention into such agents. The most straightforward is to identify drug-like small molecules that mimic the effect of Apt48. A rapid means to identify such molecules would be first to screen for those that can inhibit the binding of Apt48 to BCL-6 POZ, then to verify which of these are able to antagonise BCL-6 function.

**BCL-6 Surface**

The invention defines a novel surface on the BCL6 protein which is a target for repression of its activity. This surface is defined as the second and third strands of anti-parallel β sheet of BCL6. For example, the surface as defined in the table presented in Figure 2c. In particular, referring to row 2 of that table, the surface of interest on BCL6 is defined with reference to the sequence DILTDVVIVVSREQFRAH. This is a specific tertiary structure within the overall sequence of the POZ-domain of BCL6. Thus, the invention relates to use or targeting of this part of BCL6 in the suppression or inhibition of BCL6 activity. Furthermore, the invention relates to the screening or targeting of potential repressors to the surface.
Peptides
Preferably the peptides of the invention are discussed with relation to their core unique sequence. In many embodiments, this will be the 10mer unique peptide sequence of the particular peptides and aptamers being discussed.

Preferably, the aptamer is used as a free peptide.

In some embodiments, peptides of the invention may be fused with a scaffold together with elements used in an interaction trap cloning scheme, such as 2 hybrid scheme. Preferably, the peptides are fused only to the scaffold protein. Preferably, the peptides are incorporated into a context of the operator's choice.

The peptide sequences are given with reference to the core unique sequence, in many embodiments, these will be flanked by "GP" residues at the N and C terminal. These 2 amino acid residues are generated from the Rsr II cloning site which is used to incorporate the peptide aptamers into the particular vectors for conducting the screening and analysis presented herein. Thus, preferably the peptide aptamers comprise N and/or C terminal GP amino acid residues. Thus, a 10mer peptide may preferably comprise a core unique sequence often amino acids flanked by a GP dimer at each terminus, totalling 14 amino acid residues in length.

The invention also relates to nucleic acids encoding the peptides of the invention. In this regard, preferably such nucleic acids are in the form of nucleic acid cassettes. Preferably, these cassettes comprise a coding sequence for the unique 10 amino acid sequence of the peptide, flanked at each end by an Rsr II site. Thus, preferably overall the nucleic acid will encode 14 amino acids, i.e. a GP followed by the unique peptide aptamer sequence followed by a second GP.

When referring to a scaffold protein, preferably the scaffold protein is TrxA (thioredoxin based scaffold protein) or is STM (stefin A based scaffold protein).

The peptides of the invention are preferably used as free peptides.
The peptides of the invention are preferably used as circularised ring peptides.

The invention also embraces mimetics of the peptides disclosed herein, or other chemical equivalents thereof.

The peptides of the invention are preferably fused to supplementary peptides or proteins depending on their desired use. For example, it may be desirable to direct to the peptide to B cells or cells of the B cell lineage. In this embodiment, preferably the peptide is fused to the CD 20 ligand. This has a technical benefit of the CD 20 ligand portion of the fusion protein directing the fusion protein to the surface of B cells. In a preferred embodiment, this binding is sufficient to lead to endocytosis and internalising of the aptamer-CD20 ligand fusion protein. Thus, the invention advantageously provides a one step targeting and internalisation construct by fusion of the peptide of interest to the CD20 ligand. This can be applied equally to any other suitable ligand.

Preferably, the peptides of the invention are directed into the target cells. This may be accomplished by targeting the peptides to receptors which, upon binding, become internalised thereby accomplishing targeting and internalisation in one convenient step. Alternatively, or in addition, the peptides may be fused to transduction domains in order to achieve internalisation. Any suitable transduction domain known in the art may be employed for this purpose. Indeed, combinations or multiple transduction domains may be fused to the peptides of interest in order to achieve the desired effect if necessary. Preferably, the transduction domain would be selected from the classes of HIV tat, Drosophila antennapedia, VP22 or arginine repeat. When the transduction domain is based on the arginine repeat, preferably the domain comprises at least 8 or 9 consecutive arginine residues. Preferably, the domain comprises at least 9 consecutive arginine residues.

Preferably, the peptides of the invention are targeted to the nucleus. Such targeting may be direct, for example by use of a nuclear localisation signal, or may be indirect.
Examples of indirect targeting may include "piggy backing" by binding with a nuclear localised protein in the cytoplasm at the stage of protein synthesis, followed by translocation into the nucleus by association with that binding partner. Preferably, nuclear localisation is ensured by use of appropriate targeting domain fused to the peptide of interest. An example would be a standard nuclear localisation signal.

Preferred peptide amino acid sequences are given in the following table. Preferably the capitalised sequence is the sequence of interest.

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apt 48</td>
<td>gpHGPRDWCLFGgp</td>
</tr>
<tr>
<td>Apt 52</td>
<td>gpGGDYCCLRYVgp</td>
</tr>
<tr>
<td>Apt 58</td>
<td>gpWGGVMLVTWPgp</td>
</tr>
<tr>
<td>Apt 7</td>
<td>gpVRGYGASLLLgpPISAV</td>
</tr>
</tbody>
</table>

- **Aptamer 7**
  Aptamer 7 has been created by the internal fusion of two individual peptide cassettes. Aptamer 7 has the advantage of being very specific. Aptamer 7 has the advantage of a greater size than the 1Omer aptamers disclosed herein.

- **Aptamer 52**
  Aptamer 52 is a preferred peptide of the invention. Aptamer 52 shows a relatively non specific binding pattern. For example, aptamer 52 binds the POZ-domain of PLZF, and of BCL-6, and of BACH2. Furthermore, aptamer 52 interacts with CDK4.

  Aptamer 52 thus has the advantage of targeting many pathways simultaneously. The technical benefit of this feature is that it may offer better therapeutic effect by simultaneously intervening in multiple signalling pathways.

- **Aptamer 58**
  Aptamer 58 demonstrates a degree of functional activity. Although this activity is not prominent in the immunofluorescent experiment shown in figure 3, as will be appreciated by the skilled reader it is difficult to visualise red cells in this type of assay.
and so the modest activity read out from this assay is not a negative indication of the activity or usefulness of aptamer 58.

Aptamer 58 has the advantage of intermediate specificity.

Aptamer 58 has the advantage of being a strong binder to BCL6. Furthermore Aptamer 58 has affinity for CDK4. Both of these proteins are clinically relevant targets. Thus, technical advantages associated with aptamer 58 include the opportunity to influence multiple pathways through a single peptide.

**Sequence Variants**

Although the preferred peptides of the invention are presented in terms of their amino acid sequence, the skilled reader will appreciate that variations in the amino acid sequence will be possible. By variations is meant substitution, deletion or addition of one or more residues. Preferably, the resulting peptide should show at least 40% sequence identity to the relevant unique core aptamer sequence disclosed herein, preferably 50%, preferably 60%, preferably 70%, preferably 80%, preferably 90% and most preferably 100% sequence identity. In another embodiment, sequence comparison may be taken to mean sequence similarity, taking into account conservative substitutions within the core aptamer sequence.

In all circumstances, sequence variants of the aptamer peptides disclosed herein should retain the biological function of the reference peptide. The assays for that biological function are set out herein with specific reference to the examples.

In order to produce sequence variant peptides retaining the activity, the skilled reader can easily vary the sequence and compare the new peptide sequence with the original peptide sequence in the assays to ensure that activity has been retained. Specifically, in order to generate variants of the peptides within the invention, the skilled operator may use error prone PCR to randomise the peptide. The randomised peptides would then be screened in yeast for retention or enhancement of the binding activity. These
enhanced or retained binders would be considered biologically active variants of the peptides of the invention.

In order to determine the consensus sequence for a particular activity, the same procedure can be carried out. In this embodiment, peptides retaining or increasing their binding would be of interest, as would variants which exhibit a loss of binding. Each of the variants would then be sequenced, and sequence comparison between binders and non-binders will identify the particular residues which must be conserved, and those which may be varied. These analyses are well within the abilities of the person skilled in the art.

Preferred Features

In a preferred embodiment of the invention, the peptide of interest possesses a D-hydrophobic-C motif. For example, in aptamer 48 this motive is found as DWC. Furthermore, in aptamer 52 this motive is found as DYC.

"Aptamer" preferably has its usual meaning in the art. For the avoidance of doubt, a "peptide aptamer" as used herein means a peptide whose amino and/or carboxytermini are anchored in the context of a scaffold protein. Preferably, an aptamer is a peptide whose amino and carboxytermini are anchored in the context of the scaffold protein. If the phrase "aptamer" is used herein to describe a free peptide, then it will be understood to refer to a peptide having a unique amino acid sequence which was cloned as the corresponding constrained aptamer.

Pharmaceutical Compositions

The present invention also provides a pharmaceutical composition comprising a therapeutically effective amount of the peptide(s) of the present invention and a pharmaceutically acceptable carrier, diluent or excipient (including combinations thereof).
The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be administered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be administered by a number of routes.

Where the agent is to be administered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion,
solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For some embodiments, the agents and/or growth factors of the present invention may also be used in combination with a cyclodextrin. Cyclodextrins are known to form inclusion and non-inclusion complexes with drug molecules. Formation of a drug-cyclodextrin complex may modify the solubility, dissolution rate, bioavailability and/or stability property of a drug molecule. Drug-cyclodextrin complexes are generally useful for most dosage forms and administration routes. As an alternative to direct complexation with the drug the cyclodextrin may be used as an auxiliary additive, e.g. as a carrier, diluent or solubiliser. Alpha-, beta- and gamma-cyclodextrins are most commonly used and suitable examples are described in WO-A-91/11 172, WO-A-94/02518 and WO-A-98/55148.

When the agent is a peptide, then said peptide may be prepared in situ in the subject being treated. In this respect, nucleotide sequences encoding said peptide may be delivered by use of non-viral techniques (e.g. by use of liposomes) and/or viral techniques (e.g. by use of retroviral vectors) such that the said peptide is expressed from said nucleotide sequence.

In a preferred embodiment, the pharmaceutical of the present invention is administered topically. Hence, preferably the pharmaceutical is in a form that is suitable for topical delivery.
Administration

The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectors, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof.

The components of the present invention may be administered alone but will generally be administered as a pharmaceutical composition - e.g. when the components are in admixture with a suitable pharmaceutical excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the components can be administered (e.g. orally or topically) in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, sustained-, pulsed- or controlled-release applications.

If the pharmaceutical is a tablet, then the tablet may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably com, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the agent may be combined with various sweetening or flavouring agents,
colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The routes for administration (delivery) include, but are not limited to, one or more of: oral (e.g. as a tablet, capsule, or as an ingestable solution), topical, mucosal (e.g. as a nasal spray or aerosol for inhalation), nasal, parenteral (e.g. by an injectable form), gastrointestinal, intraspinal, intraperitoneal, intramuscular, intravenous, intrauterine, intraocular, intradermal, intracranial, intratracheal, intravaginal, intracerebroventricular, intracerebral, subcutaneous, ophthalmic (including intravitreal or intracameral), transdermal, rectal, buccal, vaginal, epidural, sublingual.

In a preferred aspect, the pharmaceutical composition is delivered topically.

It is to be understood that not all of the components of the pharmaceutical need be administered by the same route. Likewise, if the composition comprises more than one active component, then those components may be administered by different routes.

If a component of the present invention is administered parenterally, then examples of such administration include one or more of: intravenously, intra-arterially, intraperitoneally, intrathecially, intraventricularly, intraurethrally, intrastemally, intracranially, intramuscularly or subcutaneously administering the component; and/or by using infusion techniques.

For parenteral administration, the component is best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

As indicated, the component(s) of the present invention can be administered intranasally or by inhalation and is conveniently delivered in the form of a dry powder
inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A™) or 1,1,1,2,3,3,3-heptaffluoroOpropane (HFA 227EA™), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, e.g. using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of the agent and a suitable powder base such as lactose or starch.

Alternatively, the component(s) of the present invention can be administered in the form of a suppository or pessary, or it may be applied topically in the form of a gel, hydrogel, lotion, solution, cream, ointment or dusting powder. The component(s) of the present invention may also be dermally or transdermally administered, for example, by the use of a skin patch. They may also be administered by the pulmonary or rectal routes. They may also be administered by the ocular route. For ophthalmic use, the compounds can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

For application topically to the skin, the component(s) of the present invention can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, it can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a
polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Pharmaceutical Combinations

The agent of the present invention may be administered with one or more other pharmaceutically active substances. By way of example, the present invention covers the simultaneous, or sequential treatments with an agent according to the present invention and one or more steroids, analgesics, anlivirals or other pharmaceutically active substance(s) such as other BCL-6 repressors.

It will be understood that these regimes include the administration of the substances sequentially, simultaneously or together.

Dose Levels

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject. The specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy.

Depending upon the need, the agent may be administered at a dose of from 0.01 to 30 mg/kg body weight, such as from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

Formulation

The component(s) of the present invention may be formulated into a pharmaceutical composition, such as by mixing with one or more of a suitable carrier, diluent or excipient, by using techniques that are known in the art.
Pharmaceutically Active Salt
The peptide(s) of the present invention may be administered as a pharmaceutically acceptable salt. Typically, a pharmaceutically acceptable salt may be readily prepared by using a desired acid or base, as appropriate. The salt may precipitate from solution and be collected by filtration or may be recovered by evaporation of the solvent.

Treatment
It is to be appreciated that all references herein to treatment include one or more of curative, palliative and prophylactic treatment. Preferably, the term treatment includes at least curative treatment and/or prophylactic treatment.

The treatment may be of one or more of those disorders mentioned herein, or related complaint.

Therapy
The agents/modulators identified by the methods of the present invention may be used as therapeutic agents – i.e. in therapy applications. As with the term "treatment", the term "therapy" includes curative effects, alleviation effects, and prophylactic effects. The therapy may be on humans or animals. The therapy can include the treatment of one or more of those disorders mentioned herein, or related complaint.

The agents/peptides or compositions of the invention find application in lymphoma such as Burkitt's lymphoma.

The agents/peptides or compositions of the invention find application in bladder cancer, colorectal cancer, and breast cancer or any other cancer in which Bcl-6 is de-repressed.

Aptamer 48 finds application in the increase of p53 expression. Without wishing to be bound by theory, it is believed that this effect is mediated by disruption of BCL-6 repression of the p53 promoter.
Aptamer 48 finds application in the increase of Blimp-1 expression.

The invention will now be described by way of non-limiting example with reference to the following figures.

**Brief Description of the Figures**

Figure 1. *Yeast two hybrid interaction matrix for BCL-POZ, related POZ domains and the peptide aptamers isolated in the screen*. Panel A. POZ domain- containing proteins have previously been shown to interact. Haploid yeast strains carrying either the bait (DNA binding domain fusions to the POZ domains of BACH2, BCL-6b, BCL-6, and PLZF; shown in columns) or the prey (activation domain fusions; shown as rows) were mated by replica plating. Selection for diploid cells from the grid of mating haploid cells gives rise to the pattern of squares in the Figure. The interaction between CDK4 and cyclin D (Cyc D in the Figure) is a positive control for the yeast two hybrid interaction assay. Yeast strains carrying empty bait and prey fusion vectors are indicated by "-". Panel B: the expression of the peptide aptamers and cyclin D prey fusion proteins is induced by galactose present in the growth medium, allowing interactions to be measured by the development of a blue colour following induction of the LacZ reporter gene. Apt48 binds most strongly to the BCL-6 POZ and shows no significant cross-reactivity, while Apt52 and 58 appear to bind to CDK4 as well as to BCL-6 POZ. Apt7/130 is the most specific binder, but does not bind BCL-6 POZ as strongly as Apt48. Panel C: BCL-6 and Apt48 can be coimmunoprecipitated from cells. Constructs carrying Flag- and myc-tagged BCL-6 and myc-tagged Apt48 or thioredoxin were co-transfected into U2os cells. BCL-6 was immunoprecipitated from whole cell lysates (WCL) with an anti-Flag tag antibody and the immuno-precipitates (IP) probed with an anti-myc antibody. Lanes 1 and 3 are from cells co-transfected with Apt48 and BCL-6, while lanes 2 and 4 are from cells co-transfected with TrxA and BCL-6. The band marked with an asterisk that appears in the IP lanes is the antibody light chain. Panel D: Sequences of the peptide aptamers isolated in the screen. Sequences of the peptide aptamers that interact with the POZ domain of BCL-6. The hexameric restriction/ligation sequence used to insert oligonucleotides into the TrxA open reading frame encodes a glycine-proline (GP) dipeptide, given in lower case. In
the case of Apt7, an in-frame STOP codon encoded by the second of two inserted oligonucleotides results in a truncated form of TrxA protein, and the encoded peptides are presented, unconstrained, at the carboxyl terminus of the fusion protein.

Figure 2. *The mechanism of action of Apt48 may not involve displacement of the SMRT co-repressor from BCL-6 POZ.* Panel A: Sequence alignments of the SMRT BBD sequence that is known to bind to the POZ domain of BCL-6 (top line; Polo *et al.*, 2004), the unique sequence of Apt48 (middle line), the designed version of Apt48 intended to mimic SMRT ("Des": third line) and a sequence for BCL-6 with complementarity to Apt48. Identical residues are highlighted in red, including the cysteine residue that were proposed to form a cross-bridge in the Apt48-POZ complex in our first model, while residues of POZ that are complementary to residues in Apt48 are highlighted in green and the glycine-proline motif that flanks peptides inserted into the peptide aptamer scaffold is given in lower case. Panel B: Yeast two hybrid interaction matrix to show that a mutation in BCL-6 POZ (N21K) that abolishes binding to SMRT-BBD does not affect binding to Apt48 or to POZ. Mutation of other residues of POZ (E81K and C84N) to the corresponding residues present in PLZF also do not affect Apt48 or POZ binding. pJM-1 expresses *E. coli* thioredoxin and differs from Apt48 and BBD only in not carrying an inserted peptide. Panel C: Seven fragments of BCL-6 POZ corresponding to the elements of secondary structure revealed by Ahmad *et al.* (2003) were cloned as yeast two hybrid baits and their interaction with preys consisting of Apt48, the SMRT-BBD peptide aptamer or BCL-6 POZ itself was determined. Fragment 1 interacted with POZ and with BBD, as predicted by the crystal structure, but did not recognise Apt48. Fragment 2 did bind Apt48, and also recognised POZ, but not the BBD. Fragment 3 shows weak interaction with POZ but not with Apt48 or BBD. Fragment 5 was a trans-activating bait whose interaction with POZ was strong enough to be seen above the trans-activation signal, but it was not possible to determine whether there was an interaction with Apt48 or BBD (ND: not determined). Fragments 4, 6 and 7 do not interact with POZ, BBD or Apt48 in this assay.

Figure 3. *The expression of Apt48 in cells relieves transcriptional repression.* Panel A. Ramos cells normally express low levels of CD69, which are undetectable in this
immunofluorescent assay and are not increased by expression of thioredoxin (left hand panels). In contrast, cells expressing Apt48 show significant levels of CD69 expression (right hand panels). CD69 expression is shown in red, and thioredoxin or Apt48 protein is shown in green. Nuclear DNA is stained blue, and the three panels are merged in the bottom right hand image of each set. Panel B: Ramos cells transfected with thioredoxin or peptide aptamer as above were processed for immunofluorescence and the samples anonymised before being independently counted by two individuals. As the data did not differ significantly, the two sets of data were combined, and are presented as the percentage of green (peptide aptamer or thioredoxin expressing) cells that were also expressing detectable levels of CD69. Panel C. Mouse A20 (left hand pair of each set of 4) or human Ramos (right hand pair of bars) transfected with expression constructs for either Apt48 (gray bars) or thioredoxin (black bars) upstream of an internal ribosome entry site and the ORF encoding red fluorescent protein were isolated by flow cytometry, lysed and mRNA levels measured by Real Time-PCR. When normalised to the loading control (actin for mouse, GAPDH for human cells), the mRNA levels for three known BCL-6 target genes (CD69, BLIMP-I and Cyclin D2) show significant increase in cells expressing Apt48 when compared to those expressing empty scaffold (thioredoxin) alone. The increases range from 7 (CD69 in mouse A20 cells) to 15 fold (Blimp-1 in mouse cells).

Figure 4. **Apt48 can antagonise BCL-6 mediated gene repression in an exogenous system.** The interleukin dependent expression of a firefly luciferase reporter gene in transfected BCL1 cells is dependent on Stat3 binding sites, and can be repressed by BCL-6. Each pair of bars shows luciferase activity in the absence (left hand bar) and presence (right hand bar) of interleukins 2 and 5. In the absence of BCL-6, luciferase activity is induced by interleukin treatment ("vector alone") and this is not impaired by expression of Apt48 ("vector + Apt48"). Expression of BCL-6 prevents interleukin mediated induction of luciferase ("vector + BCL-6"), unless Apt48 is also expressed, in which case mterleukin-mediated induction of luciferase is restored ("BCL-6 + Apt48").
Figure 5. *Apt48 reverses BCL-6 mediated resistance to cytokine mediated growth arrest in BCLl cells*. Panels A and B: The growth of BCLl cells expressing either GFP alone (♦) or GFP and BCL-6, GFP and BCL-6 and TrxA, or GFP and BCL-6 and Apt48, was measured over three days in the absence (panel A) and presence (panel B) of interleukin 2 and 5. In the absence of these cytokines, cells expressing BCL-6 alone, or BCL-6 and TrxA appear to have a slight growth advantage at day 2, but this effect, which was not robust, is negligible by day 3. In contrast, in the presence of interleukins, cells expressing GFP alone fail to grow, while cells expressing BCL-6 alone grow exponentially. TrxA is without effect on BCL-6 mediated cytokine resistance, but Apt48 restores cytokine mediated growth inhibition on BCL-6 expressing cells. Panels C, D and E: *Apt48 inhibits the growth of BCL-6 expressing Burkitt’s lymphoma cells*. Ramos (panel C), Raji (panel D) and Daudi cells (panel E), that constitutively express BCL-6, were transfected with GFP-expression plasmid alone (♦), or GFP + either TrxA or Apt48. Although TrxA and EGFP appear to have some toxicity in this assay, as cell numbers do not increase over time and in fact decrease with TrxA expression, Apt48 expression leads to a significant decrease in cell viability, from 90% for EGFP expressing cells to 56% for Apt48 (panel C). Similar results were obtained with two other BCL-6 expressing cell lines, Raji (panel D) and Daudi (panel E).

Figure 6 shows a western blot to determine the presence of Bcl-6 protein in epithelial cancer cell lines. Cell lines tested were Cal51, HCT116+/-, HCT116-/-, SKBR7, ZR75-1, SKBR3, ZR75-13 and MDA-MB-175. The Bcl-6 protein is indicated with an arrow and is expressed in all cells tested except SKBR3.

Figure 7 shows electrophoretic mobility shift assay showing that the binding of proteins in cell extracts to either a highly specific BCL-6 binding site (ie STAT6, left hand lanes) or the more generic BCL-6 binding oligonucleotide (B6BS, right hand lanes) is not affected by expression of Apt48, the empty scaffold (Trx) or the irrelevant GFP.

Figure 8 shows chromatin immuno-precipitation and quantitative RT-PCR of promoters normally repressed by Bcl-6. Purple bars indicate the relative levels of mRNA expressed from each of the BCL-6, cyclin D2 (Ccd2), Blimp-1 and p53 promoters given as the ratio from cells expressing Apt48 to levels in cells expressing
the empty thioredoxin scaffold. A value of 1 indicates no change. Ratios indicating an increase over 1 are not deemed significant unless they exceed 2, as is the case for Blimp-1. The levels of BCL-6 (blue bars) protein present at each promoter do not change in cells expressing Apt48. N-CoR (maroon bars) recruitment to the promoters is similarly unaffected, except in the case of the Blimp-1 promoter, where its level appears to decrease by more than half in cells expressing Apt48. MTA3 levels generally decrease at promoters where expression is increased (ie all promoters tested except that for the cycliii D2 gene), although neither change was statistically significant. No consistent changes in Sin3A (light blue bars) were noted.

Figure 9 shows co-immunoprecipitation of MTA3 with anti-BCL6 antibodies is decreased in cells expressing Apt48. Nuclear extracts were prepared from Ramos cells expressing empty scaffold protein (Trx) or Apt48. 20 µg of nuclear extract was subjected to immuno-precipitation with an anti-BCL6 antibody, and the washed precipitates were subject to Western blotting. Probing with an anti-BCL-6 antibody showed that the IP was equally effective in both cases. Probing with an anti-MTA3 antibody revealed significantly less MTA3 associated with BCL-6 in cells expressing Apt48 compared to Trx, even though levels in the extracts were similar. It appears that there may be slightly more MTA3 protein in cells expressing Apt48. Levels of N-CoR, Sin3A and SMRT co-immunoprecipitated with BCL-6 were identical in cells expressing Apt48 compared to cells expressing Trx.

Figure 10 shows a yeast two hybrid interaction matrix showing that mutation of aspartic acid residue at position 33 of the BCL-6 POZ/BTB domain abolishes interaction with both Apt48 (A48) and the BPI inhibitor peptide in the context of thioredoxin, although the POZ domain still folds correctly as judged by its ability to homodimerise. The pPM-I vector expresses thioredoxin alone, showing no interaction between the empty scaffold and the POZ domain of Bcl-6.

Figure 11 shows upregulation of cell cycle proteins following viral infection.

**Examples**

**Materials and methods: Plasmids and cloning.**

DNA manipulations were performed as described by Sambrook and Russell (2001). Oligonucleotides were from Sigma-Genosys (Pampisford, UK). All enzymes were
from New England Biolabs (Hitchin, UK) and competent cells were from Stratagene (UK). Plasmid and nucleotide DNA was prepared and cleaned using the appropriate Qiagen (Crawley, UK) kits. FLAG tagged full-length BCL6 was a kind gift from Dr Paul A. Wade, Emory University school of Medicine, Atlanta. Sequences corresponding to the POZ domains of BCL-6, and BCL-6b were PCR amplified from mouse spleen cDNA, and of PLZF and BACH2 from plasmids supplied by Dr A. Zelent, Institute of Cancer Research, London and Dr J.V. Melo, Dept. of Haematology, Imperial College London, respectively. The PCR products were cloned as in frame fusions to the DNA binding domain of Escherichia coli LexA in the yeast two hybrid vectors pEG202 (bait) and pJG4-5 (prey; Gyuris et al., 1993) in frame with the Gal4 activation domain, using EcoRI sites present in both vectors. The sequence encoding full length BCL-6 where serines 333 and 334 are mutated to alanine residues was cloned into Sad and BamHI digested pCDNA3.1 myc/his + (hivitrogen, Paisley, Scotland). The coding sequence of peptide aptamer number 48 from the screen (Apt48) including the full coding sequence of E. coli thioredoxin A (TrxA) was PCR-amplified from the peptide aptamer prey vector pJM-1 (Colas et al., 1996) using a 5’ primer containing an EcoRI restriction site (5’- GGAATTCCACCATGGCTAGCGATAAAATTATTCAC) and a 3’ primer containing a Xbal site (5’- GCTCTAGAGCGGC-AGGTTAGCGTC) and cloned into EcoRI and Xbal digested pCDNA3.1B myc/his + (Invitrogen). Similarly, Apt48 and TrxA coding sequences were PCR-amplified using a 5’ primer containing an EcoRI site (5’- GGAATTCCACC-ATGGCTAGCGATAAAATTATTCAC) and a 3’ primer containing a SacII site (5’-TCCCCGCCG-GGACTAGGCGCCAGGTAGCGTGAG) and cloned into EcoRI and SacII digested pRES-DsRed2 (Clontech).

Peptide aptamer library construction and yeast two hybrid techniques

Yeast two hybrid plasmids and strains were obtained from Roger Brent (Molecular Sciences Institute, Berkeley, California, USA) and Erica Golemis (Fox Chase Cancer Centre, Philadelphia, USA). Fresh yeast stocks were purchased from Origene (Rockville, Maryland, USA). Standard yeast techniques are from Rose et al (1990) and yeast two hybrid screening was as described by Gyuris et al (1993) and Golemis
et al (1999). Library construction and designed peptide aptamers were made following the protocols of Colas et al (1996).

Antibodies, Immunoprecipitation and Western Blotting.

All primary antibodies were from Santa Cruz (CA, USA). Western blotting of mammalian cell lysates was performed using rabbit polyclonal antibodies N-3 or C-3 to BCL6. The myc epitope tag of Apt48 or thioredoxin or FLAG-tagged BCL6, expressed from pCDNA3.1 myc/his was detected using a myc-A14. Detection of Apt48 or TrxA in yeast lysates was performed using a monoclonal mouse antibody to the epitope tag derived from Influenza virus hemagglutinin (HA, using antibody F7). HRP conjugated goat secondary antibodies were from Pierce and membranes were developed with ECL reagent (Amersham, Chalfont St Giles, UK). For immunoprecipitation experiments, U2os cells transfected (using Gene-Juice, Novagen and OPTMEM, Gibco-BRL) with FLAG and myc-tagged-BCL6 and myc tagged-Apt 48 or - TrxA were lysed after 24 hours of transfection for 15 min at 4°C using a lysis buffer consisting of 0.5% Triton X-100, 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl2, and supplemented with Complete Protease inhibitor cocktail (Roche Molecular Biochemicals) and dimethyl sulphoxide (Sigma). After centrifugation at 13000 rpm for 15 min, the supernatant was incubated with 20 µl prewashed anti-FLAG monoclonal antibody M2 beads (Sigma) and left overnight. Aptamer-BCL6 interactions were detected by washing the beads 5 times with the same lysis buffer followed by eluting the proteins bound to the beads in SDS sample buffer for SDS-PAGE and subsequent western blotting with an anti-myc antibody.

Cell lines and Cell culture

U2os cells are an osteosarcoma cell lines that are not known to express BCL-6 and do not respond to cytokines. BCL1 cells are representative of immature B cells: they are cytokine responsive but do not express BCL-6. Ramos, Raji and Daudi cells are BCL-6 expressing human Burkitt's lymphoma lines. A20 cells are BCL-6 over-expressing mouse cells.

All tissue culture media and supplements were from Gibco-BRL (Paisley, UK). 2-mercaptoethanol was from BDH (Poole, UK). Ramos, Raji, Daudi and A20 cell lines were maintained in RPMI 1640 media supplemented with 10% fetal calf serum.
(FCS) (HyClone) and penicillin (100 U/mL) /streptomycin (100 µg/mL) (pen/strep, final concentrations). BCL1 cells were cultured in RPMI 1640 with 15% FCS, 10 nM HEPES, 50 µM 2-mercaptoethanol and pen/strep. U2os cells were maintained in D-MEM supplemented with 10% FCS.

5 **Transient transfection and immunofluorescence.**

Ramos cells were transfected with expression plasmids using the Nucleofector (Amaxa, Cologne, Germany) following the manufacturer's instructions. After overnight culture, transfected cells were seeded on poly-L-lysine coated cover slips and fixed using 4% paraformaldehyde for 20 min at room temperature, washed three times with phosphate-buffered saline (PBS) containing 1% bovine-serum albumin (BSA), permeabilised for 30 min with 0.2% saponin in 1% BSA-PBS, blocked for 1 hr with 10% normal goat serum, and incubated overnight at 4°C with primary antibodies. CD69 expression was visualised directly using allophycocyanin (APC) conjugated a-CD69 antibody (BD Biosciences, Cowley, UK) while peptide aptamer expression was visualised using a -TrxA primary antibody (Sigma, Poole, UK) with fluorescein isothiocyanate conjugated goat anti rabbit secondary (Alexa 488, Molecular Probes, Paisley UK). Nuclear DNA was visualised with DAPI (4',6'-diamidino-2-phenylindole). Coverslips were mounted using Vectashield (Vectorlabs, Peterborough, UK). The cells were examined by confocal laser-scanning microscopy (Zeiss LSM 510).

**Flow sorting, RNA extraction and RT PCR.**

Cell lines were transfected with peptide aptamers or empty thioredoxin scaffold expressed from mammalian expression vectors upstream of an IRES site and RFP. 5µg of plasmid DNA was transfected by means of a Nucleofector, Kit T (Amaxa) using programmes 0-17 for BCL1 and Ramos, and T-16 for A20 cells. After 24 hours, cells expressing RFP were collected by FACS for extraction of RNA and production of cDNA (Qiagen). Primers used were as follows:

- CD69 forward 5TATAACGGAAAATAGCTCTTCACATCT;
- CD69 reverse 5'TGATTAGCTTCTTTTCAGCCCAAT;
- Blimp-1 forward 5'TGGACATGAGGAGGACGCTGATATG;
- Blimp-1 reverse 5'GCGCATCCAGTTGCTTTTCTCCTCA;
- CyclinD2 forward 5'GCGTGGGAGCAGCCATCTCTGT;
CycliiiD2 reverse 5'AGGTCAACATCCCGCACGTCTG.

Actin was used as a loading control for the mouse A20 cells and GAPDH for the human cells. The mouse actin forward primer was 5'AGGTCATCACTATTGGCAACGA and mouse actin reverse 5'CACTTCATGATGGAATTGAATGTAGTT. Human GAPDH primers are from Shaffer et al. (2000) 13:199-212 and were GAPDH forward: 5'GGGCGCCTGGTCACCAGGGCTG and GAPDH reverse 5'GGGGCCATCCACAGTCTTCTG. Real-time PCR was performed using the same primers and CybrGreen to label PCR products, in an ABI Prism 7700 Sequence Detector, according to the manufacturers' instructions.

Luciferase reporter assay.
The STAT3 reporter construct and assay used in this study was exactly as described in Reljk et al (2000). Transfection experiments were performed in duplicate and repeated 3 times. The combinations of plasmids transfected in each case are given in the figure legend.

Survival assay
The effects of peptide aptamers on cell survival was tested in BCLI cells, which lack endogenous BCL-6, and the Burkitt's lymphoma cell lines Ramos, Raji and Daudi, which expresses BCL-6. Cells were transfected with combinations of plasmids constituting 5µg of DNA in total and including pEGFP (BD Clontech) as a marker of transfection using a Nucleofector (Amaxa). The plasmid combinations used were 1) BCL-6 expression plasmid + peptide aptamer expression plasmid + pEGFP; 2) BCL-6 expression plasmid + thioredoxin expression plasmid + pEGFP; 3) peptide aptamer expression plasmid + pEGFP; and 4) thioredoxin expression plasmid + pEGFP. After transfection, cells were cultured in complete medium without other additives and, in the case of BCLI cells, in medium containing interleukins 2 and 5 for up to 3 days. The proportion of viable cells expressing EGFP, a marker for transfection, was measured on a FACScalibur (BD Biosciences) and normalized relative to the value at day 1 after transfection. Ramos, Daudi and Raji cells were transfected with peptide
aptamer expression plasmid + pEGFP or thioredoxin expression plasmid + pEGFP and the effect of transfection on cell survival was measured as above.

**Example 1 Yeast two hybrid analysis of POZ domain interactions**

We wished to identify peptide aptamers that would bind to the independently folding POZ domain of BCL-6 in a yeast two hybrid screen. Our goal was to obtain peptide aptamers that bind to the POZ domain of BCL-6 but not that of other selected transcription factors (BACH2, BCL-6b, and PLZF), that are able to heterodimerise with BCL-6 (Table I), are expressed in B-cells or, in the case of BCL-6b have a virtually identical DNA binding site to BCL-6.

<table>
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<tr>
<th>Transcription Factor</th>
<th>Heterodimerises with BCL-6</th>
<th>Expressed in B cells</th>
<th>Identical DNA binding domain</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PLZF</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Okabe et al, 1998</td>
</tr>
<tr>
<td>BCL-6b</td>
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<td>Unknown</td>
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<td>BACH2</td>
<td>Unknown</td>
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In order to confirm that the BCL-6 POZ domain behaved appropriately in yeast, we asked whether we could reproduce in the yeast two hybrid system known interactions of BCL-6 POZ with these POZ domains (Figure IA). We confirmed in eukaryotic cells the previously reported homodimerisation of the POZ domains of BCL-6 and of PLZF shown in crystal structures (Ahmad et al, 1998; Melnick et al, 2000). We also observed previously unreported interactions between POZ domains (compare Figure IA and Table I). Thus BACH2 appears to interact with all the POZ domains tested, including itself and BCL-6 POZ. We also note an interaction between the POZ domains of BCL-6 and BCL-6b. Finally, we note that homodimerisation between the POZ domains e.g. of PLZF and BCL-6 is of greater strength than formation of heterodimers e.g. of BCL-6 and BACH2 or BACH2 and BCL-6b in this assay.
Peptide aptamers that bind specifically to the POZ domain of BCL-6

To screen for peptide aptamers that specifically bind the POZ domain of BCL-6, we first constructed a yeast two hybrid library of random peptides 10 amino acid residues in length using the well characterised *Escherichia coli* thioredoxin (TrxA) scaffold. Although the standard length for the peptide moiety of peptide aptamers is 20 amino acid residues (Colas *et al.*, 1996), we reasoned that a shorter peptide would give rise to more stable peptide aptamers by minimizing disruption to the scaffold. Shorter peptides may also lead to more stable peptide conformations. Both of these effects may lead to a greater efficacy in screening. We screened 250,000 peptide aptamers for their ability to bind to a bait comprising the first 122 amino acids of BCL-6. We isolated seven positive hits. We sequenced the thioredoxin/peptide open reading frame for each plasmid and identified four unique peptide sequences (Figure 1, panels B and D). One of these (Apt 7) is not fully constrained and appears to be the result of the fusion of two peptide-encoding oligonucleotides during library construction, the second of which encodes an in-frame stop codon. Its apparent high specificity for BCL-6 POZ may reflect a large surface area of interaction of the unconstrained peptide moiety, potentially giving rise to a greater number of interactions between the peptide and the target. Apt52 shows relatively weak binding to both PLZF and BACH2, as well as to the unrelated CDK4 protein (Figure IB). Apt48, 58 and 7 (which has the same sequence as Apt130) all show a high degree of specificity for BCL-6 (Figure IB). Apt58 did not show a strong activity in our first functional assay (see below). Apt48 shows the highest apparent affinity in this assay, approximating the apparent strength of interaction between BCL-6 POZ monomers (compare Figure 1A and IB. Such a comparison is possible because we transform equal amounts of plasmid into yeast in each experiment, and always include standard controls, such as the androgen receptor and the naturally dimerizing Cyclin D and CDK4, which allows us to compare signal intensities between plates. In addition, we confirm equal expression levels of each peptide aptamer in the cells by western blotting. In order to validate the yeast two hybrid interaction, we showed that Apt48 (Figure 1 panel C, lanes 1 and 3), but not thioredoxin (lanes 2 and 4) could be coimmunoprecipitated with Flag-tagged full length BCL-6 (Fujita *et al.*, 2004) from U2os cells (Figure 1C). The hit rate in our
screen (1:10^5) is identical to that observed with 20-mer peptide insert libraries. If we assume that BCL-6 POZ is a representative yeast two hybrid bait, this hit rate would indicate that, at least in yeast, thioredoxin molecules are likely to be equally stable with 10 or 20 residue peptide inserts. On the basis of these observations, Apt48 was chosen as the focus of the following further examples.

Example 2: Apt48 binds outside the known co-repressor and dimerisation interfaces

The crystal structure of the POZ domain of BCL-6 bound to a peptide derived from the SMRT co-repressor show at least two distinct BCL-6 surfaces that may be susceptible to manipulation (Ahmad et al, 2003). One of these allows the dimerisation of BCL-6 POZ, and the other allows interaction with a relatively small surface of the co-repressor. Melnick et al (2002) have suggested the existence of a third surface that forms a charged pocket whose integrity is required for BCL-6 function, but no interaction at this surface has been demonstrated unequivocally. We wished to find out whether Apt48 binds to POZ in the same manner as SMRT. Firstly, we inserted residues into the first half of the peptide moiety of Apt48, creating a designed (Des) hybrid peptide aptamer between Apt48 and SMRT BBD sequences (Figure 2A). (Because it is difficult to predict whether the inserted Des peptide, when constrained by the thioredoxin scaffold, will adopt the appropriate conformation to lie in the POZ groove revealed by Ahmad et al (2003), we inserted both one and two copies of the Des sequence into thioredoxin, rationalizing that the extra length would minimize the conformational constraints imposed by the scaffold. We were unable to detect an interaction between Des and BCL-6 POZ. In a second experiment, we inserted the BBD peptide derived from SMRT by Ahmad et al (2003) into the thioredoxin scaffold. This SMRT/BBD peptide aptamer was able to bind to BCL-6 POZ (Figure 2B) and interaction was abolished by the N21K mutation that abolishes SMRT binding to BCL-6 (Ahmad et al, 2003). Importantly, the N21K mutation was without effect on Apt48 interaction with BCL-6 POZ (Figure 2B). Together, these data lead us to conclude that Apt48 binds to POZ in a manner different from the SMRT peptide. In an initial attempt to identify the Apt48 binding site, we sought unique regions of sequence
complementarity between Apt48 and BCL-6, that are not present in PLZF or other POZ domains we had tested, with the idea that mutation of critical residues in such regions of BCL-6 would abolish Apt48 binding. The best candidate for such a region comprised residues P80 to L86 of BCL-6 POZ (Figure 2A), and our hypothesis was that E81 and C84 of BCL-6 POZ may form bonds with the arginine and cysteine residues of Apt48 (Figure ID). However, mutation of these residues in BCL-6 (E81K and C84N) failed to affect the interaction between BCL-6 POZ and Apt 48 (Figure 2B), indicating that this surface is likely not to be important in recruitment of Apt48. We therefore adopted an open-ended approach, using yeast two hybrid interaction mapping to determine which surface of BCL-6 POZ is being targeted by Apt48. We created 7 fragments of BCL-6 POZ (Figure 2C) that correspond to the main elements of secondary structure in each monomer in the crystal structure (Ahmad et al 2003). We predicted that fragment 1 (containing the first beta strand, pi and alpha helix al) would interact with both POZ and the BBD, and this was indeed what we observed (Figure 2C). Fragment 3 (a2 and a3), which interacts with the opposing monomer in the structure, also provides supporting interactions for BBD binding in the structure. We could detect a weak interaction between fragment 3 and POZ, but not between fragment 3 and BBD (Figure 2C). Fragment 1 and 3 did not interact with Apt48. Consistent with this, the N21K mutation that maps in fragment 1 does not inhibit binding of Apt48 (Figure 2B). (Figure 2C). Fragments 4 ((34), 6 (P3 J~and a5)) and 7 (a6) did not interact with POZ, BBD or Apt48 (Figure 2C). Contrary to expectations, we observed interactions between POZ and fragments 2 ((32 and (33) and 5 (a4). Fragment 2 was also able to interact with Apt48, but not BBD. Because fragment 2 is the only one that is able to interact with Apt48 in this assay, we conclude that fragment 2 is necessary and may be sufficient for Apt48 binding to POZ. In the structure, this fragment lies at the top of the monomer and contributes to the formation of the charged pocket identified by Melnick et al (2002), whereas the SMRT peptide binds in a groove at the opposite surface (Ahmad et al, 2003).
Example 3: Apt48 promotes the expression of differentiation markers in BCL-6 expressing B cell lines.

BCL-6 expression has been suggested to inhibit the terminal differentiation of B cells (Reljic et al, 2000). BCL-6 inhibition experiments suggest that BCL-6 functionally down-regulates the expression of a range of genes including CD69, CD44, BLIMP-I and cyclin-D2 (Shaffer et al, 2000). We therefore asked whether the expression of Apt7, Apt48 or Apt58 in BCL-6 over-expressing Ramos cells would affect the expression of BCL-6 target genes. In a first experiment, we took advantage of the fact that CD69 is a cell-surface protein and performed FACS analysis of Ramos cells stained with APC-labelled anti-CD69 antibodies after transfection with Apt48 or control plasmids. We consistently obtained increases in CD69 labelling in the Apt48 transfected population compared to thioredoxin controls (data not shown). To correlate these increases with the expression of peptide aptamer in the cells, we turned to immuno-cytochemistry, staining cells for both peptide aptamer or thioredoxin using an anti-TrxA antibody and for CD69, using APC-labelled anti-CD69 antibodies. This allowed us to count only cells that had been transfected with peptide aptamer. In blind experiments, we found that whereas less than 5% of cells transfected with thioredoxin expressed CD69, more than 70% of cells transfected with Apt48 were positive for CD69 (Figure 3B). It is noteworthy that the strength of induction of CD69 did not correlate with the amount of Apt48 detected in the same cell. We infer from this that low levels of Apt48 are sufficient to cause a phenotype: once BCL-6 function has been inhibited, the phenotype is not enhanced by further increasing Apt48 levels.

We extended this result to other BCL-6 target genes, Blimp-1 and cyclin D2 (Shaffer et al, 2000; Reljic et al, 2000) using both Ramos cells as before and A20 cells, derived from a mouse B-cell lymphoma, that express BCL-6 as well as activation induced deaminase (AID) and surface IgG and thus have many of the characteristics of germinal centre cells. Using reverse transcription-PCR to analyse effects of Apt48 on gene transcription, we were able to confirm that peptide aptamer expression causes CD69, Blimp-1 and Cyclin D2 iuRNA levels to increase over background in cells expressing the peptide aptamer in both Ramos and mouse A20 cells. We confirmed
these results using real-time PCR (Figure 3C). The greatest effect we observed upon expression of Apt48 was on the Blimp-1 transcript, where Apt48 induced a 15 fold greater expression of Blimp-1 in mouse cells, and a 10-fold greater increase in human cells, than did TrxA alone. CD69 transcript appeared to be the least affected by Apt48, which induced a 7-fold increase in de-repression in mouse, and an 8-fold effect in human cells. Together, these data indicate that Apt48 is able to antagonise BCL-6-mediated repression at a number of different promoters, including at genes that are markers or regulators of B cell differentiation. These data also indicate that Apt48 is able to antagonise both the human and the mouse BCL-6 protein.

**Example 4:** Apt48 functionally antagonises BCL-6 activity in a cell model of transcriptional repression

We wished to ask whether Apt48 was able to directly affect BCL-6 activity within cells. We chose to work in undifferentiated BCL1 cells that do not normally express BCL-6. This allowed us to confirm whether the effect of Apt48 was dependent upon the expression of BCL-6. We have previously shown that expression of exogenous BCL-6 in these cells is able to repress the interleukin 2/5 dependent expression of a luciferase reporter gene regulated by STAT3 binding sites (Reljic et al, 2000). Accordingly, we co-transfected cells with reporter constructs and combinations of BCL-6 and Apt48 or empty vectors (Figure 4). Cells lacking BCL-6 show a 2.5 fold up-regulation of luciferase activity when challenged with interleukins 2 and 5, regardless of whether or not they express Apt48, confirming that any effects of Apt48 on transcriptional regulation require the presence of BCL-6. Expression of BCL-6 abolishes interleukin-mediated reporter gene induction. BCL-6 mediated repression of reporter gene induction is reversed by co-expression of Apt48, indicating that this peptide aptamer is able to inhibit BCL-6 function in this system.

**Example 5:** Apt48 restores growth arrest on BCL-6 expressing B cells

An important observation has been that BCL-6 expression correlates with the proliferation of undifferentiated B cells in germinal centres, and BCL-6 expression has
been suggested to inhibit terminal differentiation of B cells (Shaffer et al, 2000). A key test for our peptide aptamers was therefore that it be able to confer on BCL-6 expressing, proliferating B cells the ability to growth arrest in response to cytokines. Accordingly, we transfected BCL1 cells with a plasmid encoding EGFP (as a marker for transfection) either alone, or with BCL-6, or with BCL-6 and either thioredoxin or Apt48 (Figure 5, panels A and B). Three days after transfection, the numbers of GFP-expressing cells was determined, and representative curves of three independent replicates are shown in Figure 5 (panel A). These data indicated that neither BCL-6 nor Apt48 affects the growth or survival of BCL1 cells in the absence of signals that normally regulate B cell proliferation or differentiation, in contrast, when transfected cells were treated with cytokines that, in vivo, are thought to promote B cell differentiation (or apoptosis of imselected B cells), cells expressing EGFP alone arrested, while cells expressing BCL-6 did not, and indeed appeared to undergo more than two rounds of population doubling (Figure 5, panel B). These data are consistent with an inhibition of differentiation by BCL-6. The combination of thioredoxin with BCL-6 did not alleviate this effect, also allowing cells to undergo more than two rounds of population doubling in three days. In contrast, Apt 48 completely abrogated the effect of BCL-6, making BCL1 cells once again sensitive to cytokine signalling (Figure 5, panel B).

Example 6: Prevention Of Proliferation Of Lymphoma Cells

In this example we demonstrate that Apt48 can prevent the proliferation of MYC-over-expressing, germinal centre derived Burkitt's lymphoma cell lines that express BCL-6. For this experiment, we used Ramos (BCL-6 expressing, EBV-; Figure 5, panel C), Daudi and Raji cells (the latter two cell lines also express BCL-6 and are infected with EBV; Figure 5 panels D and E respectively). The cells were transfected either with a GFP expression plasmid, as a marker for transfection, or with GFP and thioredoxin (the empty peptide aptamer scaffold) or with GFP and Apt48. Although both GFP and thioredoxin transfections have some effect on cell viability, Apt48 was effective at inhibiting the growth of BCL-6 expressing cells, achieving greater than
40% reduction in cell number compared to controls in each cell line over the three
days of the experiment.

Together, these data strongly suggest that Apt48 can interfere with BCL-6 function. In
the light of the identification of Apt48 as a specific yeast two-hybrid interactor with
the POZ domain of BCL-6, of the fact that Apt48 only affects transcription in cells that
express BCL-6, and only affects cell viability in the presence of cytokines, we infer
that Apt48 is mediating its effects by interfering directly with BCL-6 function.

Example 7: Applications in Cancers

BCL-6 is a transcriptional repressor that regulates B cell differentiation, and whose
over-expression leads to abnormal proliferation and lymphoma. BCL-6 may play a
role in breast cancer, as it is expressed in high grade lesions. We have identified a
peptide aptamer inhibitor of BCL-6, called Apt48. Expression of Apt48 in BCL-6
over-expressing cells (i) de-represses BCL-6 target genes such as CD69, cyclin D2 and
Blimp-1; (ii) prevents BCL-6 mediated repression of a luciferase reporter gene
controlled by cytokine-inducible operators and (iii) prevents the proliferation of a
variety of tumour-derived BCL-6 over-expressing cell lines, including MYC-over-
expressing Burkitt's lymphoma lines.

Surprisingly, Bcl-6 de-repression by loss of a specific microRNA has been implicated
in bladder cancer, as well as colorectal cancer. Because Bcl-6 has been shown to
repress p53 expression and antagonise p53 function by inhibiting its target proteins,
including p21 in B cells, up-regulation of Bcl-6 in any cell, including non-B cells, may
be a key step on the path to tumourigenesis.

We have shown by western blotting that Bcl-6 is expressed in colorectal cancer line,
and in 5 out of 6 breast cancer lines tested (Figure 6). Our data demonstrate that the
peptide aptamer inhibitor we have identified for lymphoma may be broadly applicable
in epithelial cancers also.
Example 8: Inhibition of BCL-6

We disclose that Apt48 is a BCL-6 inhibitor. Another, called BPI, appears to inhibit BCL-6 activity at promoters involved in the regulation of cell proliferation, whereas Apt48 appears to promote the expression of genes that promote B cell differentiation, notably in de-repressing the expression of Blimp1. The effects of Apt48 mirror those of a dominant negative Bcl-6 mutant that lacks the DNA binding domain. One explanation for this could be that Apt48 destabilises Bcl-6. Bcl-6 is known to be degraded via ubiquitin-mediated proteolysis, but we have found no evidence that Apt48 destabilises Bcl-6. Another possibility is that Apt48 inhibits Bcl-6 binding to DNA. We have therefore investigated whether Apt48 inhibits DNA binding by Bcl-6 in two different assays. In the first, we monitored the effect of Apt48 on the binding of Bcl-6 to oligonucleotides encoding one of two Bcl-6 recognition sites in an electrophoretic mobility shift assay (EMSA). We used the 1e STAT6 element, or the consensus Bcl-6 binding site ("B6BS" - Figure 7). Cells transfected with TrxA or Apt48 expression constructs were lysed, and mixed with a vast excess of labelled oligonucleotides to allow proteins to recognise and bind the oligonucleotides. After incubation, the samples were run on agarose gels to separate free oligonucleotides from that to which proteins in the lysates had bound. The 1e binding site is highly specific for STAT6 and Bcl-6, and hence only a small proportion of the oligonucleotide is shifted in the EMSA. The B6BS binding site is much less specific, as shown by the large fraction that is shifted by proteins recruited from cell lysates (these proteins are likely to comprise a range of STATs as well as Bcl-6). Most importantly, in neither case did TrxA or Apt48 expression prevent the shift, indicating that Apt48 does not prevent direct binding of Bcl-6 to DNA.

In the second assay, we used chromatin immuno-precipitation to detect the binding of Bcl-6 (and some of its co-repressors) to specific target promoters (Figure 8). In this experiment, we also used quantitative RT-PCR to monitor the amount of mRNA that was being produced from each promoter (right hand, deep purple bar of each set). The promoters we interrogated were BCL-6 itself, cyclin D2 (CCD2), Blimp-1 and the p53 promoter. Data are presented as the ratio of the quantity of each promoter precipitated by each antibody in cells expressing Apt48 over the amount precipitated in cells
expressing TrxA; thus, a ratio less than one would indicate that Apt48 inhibits promoter binding by the protein targeted in the chromatin immuno-precipitation. Surprisingly, only one mRNA (that encoding Blimp-1) was significantly increased in this experiment, although it appears that Apt48 may increase p53 expression, which is consistent with recent data showing that BCL-6 can repress the p53 promoter, and would do so functionally in B cells undergoing somatic hyper-mutation. Apt48 does not seem to inhibit BCL-6 binding to the BLIMPI promoter, but levels of both MTA-3 and N-CoR appear decreased. However, this pattern is not repeated at other promoters— at the p53 promoter, for example, N-CoR recruitment is not affected, although MTA3 may be. Because MTA-3 is thought to bind to the central domain of BCL-6, rather than the POZ domain to which both Apt48 and N-CoR bind, and because Apt48 appears to be able to displace MTA3, these data suggest a model where the POZ/BTB domain of BCL-6 would recruit N-CoR to target promoters; this complex would then recruit MTA-3 to the core domain of BCL-6, perhaps via some as yet unidentified co-factor. Apt48 would inhibit an essential step in the formation of this complex, preferentially displacing MTA3. Of the known co-repressors that mediate the effects of BCL-6, only MTA-3 is known to be required for the repression of Blimp-1, which fits with our observation that Apt48 is the only known BCL-6 inhibitor to increase Blimp-1 expression.

We further investigated this model using co-immunoprecipitation experiments from nuclear extracts from RAMOS cells expressing either Trx or Apt48. In these experiments, we immuno-precipitate endogenous BCL-6, and probe the western blots with antibodies against Sin3A, N-CoR, MTA3 and SMRT. The levels of Sin3A, N-CoR and SMRT co-immunoprecipitated with aiiti-BCL-6 antibodies was not altered in Apt48 expressing cells compared to those expressing the empty thioredoxin scaffold. In contrast, we were able to show that MTA3 protein was significantly decreased in the co-ip from cells expressing Apt48 (Figure 9), while the total level of MTA3 in the nuclear extract of these cells was, if anything, slightly increased compared to cells expressing Trx. These data are consistent with our model that Apt48 binding to BCL-6 POZ interferes with events in the distant central domain.
Example 9: Application to drug discovery.

We continued to map the area of interaction of Apt48 on Bcl-6. We have shown that an 18 amino acid residue sequence at the top of the POZ domain is sufficient for Apt48 binding. An aspartic acid residue (D33) of this stretch contributes charge to the so-called charged pocket, and mutation of this residue has been shown to inhibit co-repressor binding in vitro, and to prevent transcriptional repression in the context of full length Bcl-6 in vivo. We have mutated the same residue in the POZ domain, and show by yeast two hybrid that this prevents binding both to the BPI peptide derived from the co-repressor SMRT and that it inhibits binding by Apt48. In contrast, another amino acid (R44) at the other end of the 18-residue stretch is dispensable for Apt48 and BPI binding (Figure 10), although both residues are close in space in the three dimensional structure. Thus, the D33 residue is required both for Bcl-6 activity and for Apt48 binding. Thus the invention relates to polypeptides in which the D33 residue is mutated or deleted, particularly as a control peptide.

The invention facilitates a crystal structure of an Apt48/BCL-6 POZ complex, such as via the expression of these proteins in E. coli.

We have established a fluorescence polarisation (FP) assay using FITC-labelled Apt48 and unlabelled Bcl-6 POZ, using proteins expressed in E. coli. Our first estimate of the affinity constant for the interaction between Apt48 and Bcl-6 was 2.5 μM, while follow-up data indicate that it may be as low as 300 nM, while the binding of Trx to Bcl-6 POZ in the same assay is in the micromolar range. The KD we obtained is in the published range for peptide aptamer/target interactions, and coincidentally is similar to the KD of antibodies selected during a primary response. More importantly, this KD is sufficiently low that it should allow competition by a small molecule in screens, enabling a drug displacement screen approach to drug discovery.

This assay is useful in detection of BCL-6 interactors.
**Example 10: Direct application to colorectal cancers**

Apt48 may be applied directly in colorectal cancer. The technical advantage of this is that the lower intestine is poorly served with lymphatics, so is not under the most rigorous surveillance by the immune system. Thus it is expected that a protein of bacterial origin such as thioredoxin may not be immunogenic in such an environment, or at least would not trigger an immune response, and could thus advantageously be applied directly to colorectal tumours. Indeed, without wishing to be bound by theory, since thioredoxin is a protein from *E. coli*, which is part of the intestinal flora, the peptide aptamer may not be recognised by the immune system, thereby avoiding or ameliorating problems associated with immune reduction of the therapeutic.

Apt48 appears to completely eliminate transfected HCT16 colorectal cancer cells. This result is at least as compelling as the demonstrations with B cells. Thus, it is shown that Apt48 may itself be administered as a therapeutic.

**Example 11: Further application to non-B-cell derived cancers**

Further to example 10, in the experiment shown in figure 11 equal amounts of lysates of two cervical carcinoma cell lines (C33A, and W12 at early- pLO- and late -p33-passage) were subject to SDS-PAGE and western blotting to probe for the expression of actin (loading control), the two key cyclin-dependent protein kinases CDK2 and CDK4, and the transcriptional repressor BCL-6, hitherto believed to be important only in B cells. C33A cells are not infected with virus, and express low levels of CDK2, but undetectable levels of CDK4 or BCL-6. The HPV infected (but non-carcinogenic) W12pLO cells show induced expression of both CDK4 and BCL-6, as well as increased expression of CDK2 compared to C33A cells. All three proteins appear to be still further up-regulated by passage 33 in W12 cells, where the virus has integrated into the genome and thus caused the cells to become carcinogenic.
REFERENCES


All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.
CLAIMS

1. A polypeptide comprising at least six contiguous amino acid residues of the amino acid sequence of one or more of

(i) HGPRDWCLFG, or
(ii) GGDYCCLRYV, or
(iii) WGGVMLVTWP, or
(iv) VRGYGASLLLGPPISAV, or
(v) an amino acid sequence having at least 70% identity to the sequence of any one of (i) to (iv) across the full length of said sequence.

2. A polypeptide according to claim 1 wherein said peptide comprises the full amino acid sequence of one or more of

(i) HGPRDWCLFG, or
(ii) GGDYCCLRYV, or
(iii) WGGVMLVTWP, or
(iv) VRGYGASLLLGPPISAV.

3. A polypeptide according to claim 1 or claim 2 wherein said sequence is HGPRDWCLFG (Aptamer 48).

4. Use of a polypeptide according to any of claims 1 to 3 in the expression of a BCL6 repressed gene.

5. Use of a polypeptide according to any of claims 1 to 3 in the inhibition of BCL6.

6. Use of a polypeptide according to any of claims 1 to 3 in the blocking of the POZ site of BCL6.
7. A method of inducing expression of a gene selected from the group consisting of CD69, blimp 1, and cyclin D2 in a cell by contacting said cell with a polypeptide according to any of claims 1 to 3.

8. A method according to claim 7 wherein the cell is a cell of the B cell lineage.

9. A method of inhibiting the effects of BCL6 overexpression comprising contacting a cell overexpressing BCL6 with a polypeptide according to any of claims 1 to 3.

10. A method of inducing differentiation of a proliferating B cell comprising contacting said B cell with a polypeptide according to any of claims 1 to 3.

11. A polypeptide according to any of claims 1 to 3 for use as a medicament.

12. A polypeptide according to any of claims 1 to 3 for use in the treatment of a lymphoproliferative disorder.

13. Use of a polypeptide according to any of claims 1 to 3 in the manufacture of a medicament for the prevention or treatment of a lymphoproliferative disorder.

14. A method for causing growth arrest of a cell over expressing BCL6 comprising contacting said cell with a polypeptide according to any of claims 1 to 3 and at least one cytokine.

15. A polypeptide comprising an amino acid sequence selected from the group consisting of Aptamer 7, Aptamer 48, Aptamer 52 and Aptamer 58.

16. A polypeptide consisting of DILTDVVIVSVREQFRAH.

17. A polypeptide comprising DILTDVVIVVSREQFRAH for use as a medicament.

19. Use of a polypeptide comprising DILTDVIVVSREQFRAH in the manufacture of a medicament for the prevention or treatment of a lymphoproliferative disorder.
### Fragment number | Sequence | Interaction with POZ | Interaction with Apt48 | Interaction With BBD
--- | --- | --- | --- | ---
1 | GASPADSCIQFTRHASDVILNRLNRLSRR | + | - | +
2 | DILTDVIVVSREOFRAH | + | + | -
3 | KTVMACSLGFYFSIFT | +/- | - | -
4 | DQLKCNLSVINL | - | - | -
5 | DPEINPEGFICLLDFMY | + | ND | ND
6 | TSRLNLREGNMAMMATAMY | - | - | -
7 | LGMYHVDTCRKFIKASE | - | - | -

**KEY:** α-helix, β-strand, random coil

**Figure 2**
Figure 3
Figure 6

Cal51  HCT+  HCT-  SK7
n   c  n   c  n   c  n   c

ZR1  SK3  ZR13  MDA175
n   c  n   c  n   c  n   c
Figure 7
Figure 8

ChIP for A48 v Tr2

Fold recruitment

Bcl-6  Ccd2  Bllmp-1  p53

BCL6  NCOA  MTA3  SNF3A  RNA level
Figure 9
Figure 10
Figure 11

C33A  W12p10  W12p33

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CDK4
Bcl-6
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<151> 2005-11-18

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INTERNATIONAL SEARCH REPORT

INTERNATIONAL application No
PCT/GB2006/004231

A. CLASSIFICATION* SUBJECT MATTER
INVENTION: C07K7/06 C07K7/08 C07K14/47 A61K38/08 A61K38/10

According to International Patent Classification (IPC) and to both national classification and IPC.

B. DOCUMENTATION SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A

POLOMETAL: "Specific peptide interference reveals BCL6 transcriptional and oncogenic mechanisms in B-cell lymphoma cells"
NATURE MEDICINE,
vol. 10, no. 12,
7 November 2004 (2004-11-07), pages 1329-1335, XP002416611
doi: 10.1038/nm1334
cited in the application abstract; figures 1, 2

-/--

Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents:

1. document defining the general state of the art which is not considered to be of particular relevance
2. earlier document but published on or after the international filing date
3. document which may throw doubts on priority Claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
4. document referring to an oral disclosure, use, exhibition or other means
5. document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search
25 January 2007

Date of mailing of the international search report
28/03/2007

Name and mailing address of the ISA
European Patent Office, P B 5818 Patentlaan 2
NL 2280 HV RUSSEL VAL\Tel (+31-70) 340-3040, Tx 31 651 epo nl, Fax (+31-70) 340-3016

Authorized officer
Schmidt, Harald
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### Box II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **□** Claims Nos. because they relate to subject matter not required to be searched by this Authority namely:
   
   Although claims 7-9 and 14 encompass a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. **□** Claims Nos. because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically

3. **□** Claims Nos. because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 64(a)

### Box III  Observations where unit/ of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. **□** As all required additional search fees were timely paid by the applicant, this International Search Report covers all claims.

2. **□** As all searchable claims could be searched without effort Justifying an additional fee, this Authority did not invite payment of any additional fee.

3. **□** As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. **□** No required additional search fees were timely paid by the applicant Consequently this International Search Report is restricted to the invention first mentioned in the claims; It is covered by claims Nos.:

   1-15 (all, partial, ly)

Remark on Protest

- **□** The additional search fees were accompanied by the applicant’s protest
- **□** No protest accompanied the payment of additional search fees
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15 (all partially)
   polypeptides, methods and uses directed to polypeptide (i) of claim 1

2. claims: 1,2,4-15 (all partially)
   polypeptides, methods and uses directed to polypeptide (ii) of claim 1

3. claims: 1,2,4-15 (all partially)
   polypeptides, methods and uses directed to polypeptide (iii) of claim 1

4. claims: 1,2,4-15 (all partially)
   polypeptides, methods and uses directed to polypeptide (iv) of claim 1

5. claims: 16-19 (completely)
   polypeptide and use directed to the polypeptide of claim 16
## INTERNATIONAL SEARCH REPORT

**PCT/ISA/210**

### Patent document cited in search report

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|                 |                 | GB 2422845 A            | 09-08-2006      |
|                 |                 | US 2006057123 A1        | 16-03-2006      |

| WO 2005092030   | 06-10-2005      | NONE                    |                 |
| US 6699703      | 02-03-2004      | US 7074914 B1           | 11-07-2006      |
| US 2005136404 A1| 23-06-2005      |                         |                 |

Form PCT/ISA/210 (patent family annex) (April 2006)