Protein-protein interaction & transmission of information

- what are the critical factors for communication in the cell?

- Location, location, location
- Timing
- Multiple (redundant) signaling cascades susceptible to feedback inhibition and stimulation
- Second messenger-protein & protein-protein interaction
There are very few reports of drug-like molecules disrupting protein–protein interfaces. Protein-protein binding interfaces are often large (>10 Å) and discontiguous. Homodimers in particular often bind to each other with subnanomolar affinities.

It is often stated that a small molecule would require matching the affinity of the protein to successfully compete for binding to its site. This assumption is false.

For the simplest case of inhibitor I competing with protein P2 for binding to protein P1, the fraction of P1 bound to P2 can be expressed in an equation analogous to that describing competitive enzyme inhibition. Plotting this fraction as a function of added inhibitor yields a typical maximal effect. How well the ligands compete for P1 is determined not by their relative binding constants but by the ratio of each ligand’s concentration to its own binding constant. This is true for all values of Kp and Ki. In other words, even a protein with a picomolar binding constant will be unable to compete with a weakly binding small molecule if the concentration of the protein is very low and the small-molecule concentration is relatively high. Of course, the limited solubility or bioavailability of many small molecules and their potential toxicity are often practical reasons to prefer higher affinity drug leads.
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P_1 + P_2 + I \xrightarrow{K_i} P_1 \cdot I + P_2
\]

\[
P_1 \cdot P_2 + I
\]

\[
K_i = [P_1] [I] / [P_1 \cdot I]
\]

\[
K_p = [P_1] [P_2] / [P_1 \cdot P_2]
\]

\[
\text{fraction of } P_1 \text{ in complex} = [P_1 \cdot P_2] / [P_1]_{\text{total}}
\]

\[
= ([P_2] / K_p) / (1 + [I]/K_i + [P_2]/K_p)
\]

**ratio of protein-complexed P1 vs total P1 as a function of inhibitor concentration**

![Graph showing the ratio of protein-complexed P1 vs total P1 as a function of inhibitor concentration.](image-url)
The sensitivity of protein–protein interactions in vivo will probably vary depending on both the levels of the proteins present and how they are distributed in the organism, and it may be critical to target the correct protein of the pair for maximal effect.

Analysis of signaling cascades has indicated that the net output of a network may be more sensitive to small changes in effector concentration (or perhaps percent inhibition) than an individual protein component. In other cases, a system may respond over a wide range of effector concentrations.

Caspases & Apoptosis

Programmed cell death, or apoptosis, is the regulated elimination of cells. This event occurs naturally during the course of development, as well as in many pathological circumstances that require cell death for the benefit of the organism. The deliberate elimination of cells occurs in a morphologically distinct manner that suggests an active, gene-directed process.

Investigation of the pathways involved in apoptosis provide a fascinating exercise in unraveling complex gene interactions.
To ensure survival of the organism, cells must die.

**Growth & Differentiation**

- Neurons only survive if they contact another neuron or muscle cell.
- Body cavities are formed by getting rid of cells.
- Autoreactive lymphocytes are disposed of during differentiation.

**Protection**

- Suppression of viral infection.
- Tumor suppression (absence of survival factors).
- Self-inflicted damage (e.g., free radicals).

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**Initiation of apoptosis**

**“Extrinsic Pathway”**

- Death Ligands → Death Receptors → Initiator Caspase 8 → Effector Caspase 3 → R.I.P.

**“Intrinsic Pathway”**

- DNA damage & p53 → Mitochondria/Cytochrome c → Initiator Caspase 9
Brothers in crime in cytochrome c-mediated apoptosis

**Apoptosis-activating factor 1**

- Also known as Apaf-1
- Possesses a caspase recruitment domain (CARD) which binds to prodomain of caspase-9
- Apaf-1 also binds ATP & cytochrome c - when these are bound, caspase-9 is activated

Sanctuary: Control of the Fateful Decision by the Bcl-2 Protein Family

Some family members promote cell survival, e.g. **Bcl-2** itself (B-cell lymphoma): aberrant overexpression of this protein leads to tumor formation

*C. elegans* homologue is CED-9

Other family members can promote cell death, e.g. **Bax**

Family members can dimerize with one another
Heterodimerization of pro- and anti-apoptotic family members may nullify each other’s effects

_Ultimate fate may depend on the excess of one over the other - provides more flexible control over whether the cell will die or not_
Interactions between Bcl-2 family members

Cleft for binding other Bcl family members via their BH3 helices

BH2 helix
BH3 helix of binding partner
BH3 helix

Mode of action of Bcl-2 members

Suppression of Apoptosis

- Bcl-2 binds Apaf-1, preventing its activation of procaspase-9 (All 3 bound together)
- May also prevent leakage of cytochrome c from mitochondria (some Bcl-2 is anchored to the mitochondrial outer membrane)

Activation of Apoptosis (e.g. Bax, Bad)

- Binding & neutralizing of apoptosis suppressors, e.g. Bcl-2
- Binding to mitochondria and triggering of cytochrome c release
The mitochondrial connection:
Numerous cell-death stimuli work through the mitochondrion. They cause pro-apoptotic members of the BCL-2 family, such as BAX and BAK, to either open new pores or modify existing channels in the mitochondrial membrane, releasing cytochrome c and other proteins that lead to caspase activation and cell death. BCL-2 itself, which is antiapoptotic, somehow blocks the pore or channel opening.

Defects in apoptosis contribute to many major diseases. Too much apoptosis has been linked to nerve cell loss in conditions such as stroke and Alzheimer's disease, and too little to cancer and autoimmune disease. And knowing exactly how to turn apoptosis on or off is key to developing drugs to treat diseases in which it goes awry.


NMR STRUCTURE OF BCL-XL/BAK PEPTIDE COMPLEX
M. Sattler et al. SCIENCE 1997, 275, 983.
Hamilton et al. based their design on the crystal and solution structures of Bak/Bcl-xL complex, which show the helical Bak-peptide binding into a hydrophobic cleft formed by the BH1-BH3 domains of Bcl-xL (Figure 2A). From alanine scans of the Bak-peptide it is clear that four hydrophobic residues (Val74, Leu78, Ile81, Ile85) along one edge of the helix are involved in binding. In addition, Asp83 forms an ion pair with a lysine residue of Bcl-xL. A related 26-mer peptide derived from the Bad-protein binds better to Bcl-xL, exploiting larger hydrophobic residues (Tyr, Phe) to induce a slight structural change in the binding region of Bcl-xL. Furthermore, it has been shown that the α-helix propensity of these peptides is decisive for strong binding to Bcl-xL.

On the basis of these structural requirements, Hamilton et al. designed a series of terphenyl molecules (1, 3-5) containing alkyl or aryl substituents on the three ortho positions (to mimic the key hydrophobic substituents on the helical exterior of Bak or Bad) and carboxylic acid substituents on either end (to mimic the additional ion pair).
Scheme 1. Synthesis of the benzyl substituted terphenyl derivative 3.

Scheme 2. Synthesis of the naphthylmethyl substituted terphenyl derivative 4.
Scheme 3. Synthesis of the terphenyl derivative 5.

NMR Spectroscopy. $^1$H/$^1$N HSQC spectra were recorded on a Bruker Avance DPX-500 spectrometer. The concentrations of Bcl-x$_L$ and 4 used for the experiment were 365 and 460 μM, respectively, in 10% DMSO/D$_2$O (25°C, 10.0 mM PBS, pH 7.0).

Figure 1. $^1$H/$^1$N HSQC spectra of Bcl-x$_L$ and 4 (red = Bcl-x$_L$ alone, blue = Bcl-x$_L$ + 4).
The binding affinity of these molecules for Bcl-xL was assessed by a fluorescence polarization assay using fluorescein-labeled 16-mer Bak-peptide. Displacement of this probe through competitive binding of the terphenyl into the hydrophobic cleft of Bcl-xL would lead to a decrease in its fluorescence polarization which in turn could be related to the known affinity of the 16-mer Bak/Bcl-xL complex. This assay showed (Figure 3) that the terphenyl molecule with two carboxylic acids and the isobutyl, 1-naphthalamethylene, isobutyl sequence (4) shows the strongest binding to Bcl-xL with a KD value of 114 nM. The less hydrophobic terphenyls (1 and 3) show lower affinity (KD = 2.09 and 1.89 M, respectively), emphasizing the importance of hydrophobic interactions for binding to the recognition cleft in Bcl-xL. Scrambling the position of the substituents, as in 5, leads to a significant loss in binding affinity (KD = 2.70 M), suggesting an effective shape complementarity for 4, as in the natural peptide. The importance of the hydrophobic groups is further confirmed by the weak binding of an analogue of 4 lacking the naphthyl and two isobutyl substituents (7, KD = 27.4 M). Finally, the role of the carboxylate groups was probed by partial removal (as in 6, KD = 6.8 M) or conversion to positively charged groups (as in 2 and 3b KD = 13.7 M), leading in both cases to significant loss of activity.

Docking studies with the AutoDock program using the Bcl-xL conformation found in the complex with Bak showed an optimal location for 4 in the same hydrophobic cleft as the helical peptide but in a slightly different orientation (Figure 2B). Further support for this binding site came from HSQC NMR experiments with 15N-labeled Bcl-xL protein. Addition of 4 (1.2 equiv, 460 M) led to shifts in a number of residues on the surface of Bcl-xL (F146, L130, I140, R139, W137, E193, S203, Y195, A104) near the predicted binding site. These affected residues all lie in the shallow cleft on the protein into which the Bak helix binds. Overlay of 4 and the Bak peptide within the binding pocket suggests that the terphenyl is indeed mimicking the cylindrical shape of the helix with the substituents making a series of hydrophobic contacts with the protein (Figure 2C).

In conclusion, a strategy of helix mimicry based on a substituted terphenyl scaffold was successfully applied to the design of a Bcl-xL antagonist with binding affinity in the lower nM region. Previous small-molecule inhibitors of Bcl-xL that were discovered by screening of large libraries, or by serendipity, only have KD values in the µM range. On the basis of the promising in vitro results for the inhibition of Bcl-xL by 4, preliminary cell studies on breast cancer cells are currently under investigation.
Sample Test Question. Analogous to the strategy used by Hamilton et al. for the development of a potent Bcl-\(x_L\) antagonist based on \(\alpha\)-helix mimicry, design and synthesize (be precise about synthetic steps and reagents) a non-peptidic mimic of the sequence SREWF that could be functioning as a ligand-specific antagonist at the tamoxifen-activated ER\(\alpha\). What could be fundamental problems with this specific approach for the development of a new pharmaceutical for the treatment of tamoxifen-refractory breast cancer?