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SIMILARITY BETWEEN THE SEQUENCES OF TAXOL-SELECTED PEPTIDES AND THE DISORDERED LOOP OF THE ANTI-APOPTOTIC PROTEIN, BCL-2

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The anti-cancer drug taxol is known to bind to and induce the polymerization of tubulin and has recently been shown to bind to the anti-apoptotic protein Bcl-2, but not to its homolog, Bcl-X_L. Libraries of random peptides displayed on the surface of a bacteriophage were screened to select those exhibiting affinity for taxol. The sequences of these peptides were compared to sequences of proteins involved in mitosis and apoptosis. No significant similarities were detected between the sequences of tubulins and the taxol-selected peptides. However, a high level of similarity exists between the selected peptides and the disordered loop of Bcl-2. Conversely, there was little similarity between the sequences of the selected peptides and Bcl-X_L. These results indicate that peptides displayed on the surface of a bacteriophage can mimic the ligand-binding behavior of a disordered protein loop and that comparison of the sequences of affinity-selected peptides with protein sequences can be predictive for ligand binding.

1. Introduction

Taxol is a widely used anti-cancer agent with significant anti-mitotic and apoptotic activity. Its anti-mitotic behavior derives from its binding to tubulin with subsequent induction of tubulin polymerization, stabilization of microtubules, halting of microtubule dynamics and shut-down of mitosis (Schiff et al., 1981; Jordan et al., 1993). Its apoptotic behavior was long believed to be a secondary effect stemming from this anti-mitotic action and thus also dependent upon its polymerization of tubulin (Haldar et al., 1996). However, in a number of cellular systems it has been possible to separate the anti-mitotic and apoptotic actions of taxol (Milross et al., 1996; Lieu et

al., 1997), suggesting the existence of a second molecular target for the drug. In addition, it has recently been shown that taxol binds to human Bcl-2 (Rodi et al., 1998) an anti-apoptotic protein that is known to be phosphorylated and inactivated in cells treated with taxol (Haldar et al., 1995; 1996; 1997).

Taxol-induced polymerization of tubulin takes place through the interaction of taxol with β-tubulin. Polymerization is sufficiently fast as to make it difficult to observe the interaction of taxol with tubulin heterodimers. The three-dimensional structure of zinc-induced tubulin sheets with bound taxol has recently been elucidated using electron microscopy (Nogales, et al., 1998). In that structure the taxol is in contact with several loops of β-tubulin including at least some residues in the C-terminal half of helix 1 (residues 15-25); in the helix5-helix6 loop (212-222) and in the vicinity of the main interaction with the taxane ring at leucine 275. The limited resolution of this study has precluded, as of yet, an exact accounting of the residues involved in the taxol contacts. Although the binding of taxol may be somewhat different in microtubules than in zinc-induced sheets, these observations are consistent both with the results of photocrosslinking experiments and the sequences of taxol-resistant mutants of tubulin, suggesting that taxol binding to the sheets is very similar to that in microtubules.

The binding of taxol to human Bcl-2 occurs at pharmacological levels of the drug with an apparent K_D of approximately 400 nM (Rodi et al., 1998). Bcl-X_L, a homolog of Bcl-2, has much lower affinity for taxol, with a dissociation constant at least 2 logs higher than Bcl-2. The three-dimensional structure of Bcl-X_L has been solved using x-ray crystallography and solution NMR (Muchmore, et al., 1996; Aritomi et al., 1997). That protein is made up largely of a seven-helix bundle with a 53 amino acid flexible loop (residues 28 to 80) that is not visible in either the x-ray or NMR structures. Negative NOE values are observed for most of the loop (residues 35-78) indicating that this region is highly mobile in solution relative to the overall motion of the molecule. The sequence of Bcl-2 is highly homologous to that of Bcl-X_L except in this loop region, and the three-dimensional structure of Bcl-2 is assumed to be very similar (Evans and Mansel, 1995; Craig, 1995). Functional studies have indicated that the flexible loop region is an important regulatory region for both proteins (Chang et al., 1997; Fang et al., 1998). In cells treated with taxol, at least four hydroxyl groups within and adjacent to the loop region of Bcl-2 are phosphorylated (Ito et al,., 1997; Haldar et al., 1998). In addition, proline 59 has been identified as a 'hot spot' for mutations that suppress the taxol-induced phosphorylation and subsequent inactivation of Bcl-2 (Aime-Sempe et al., 1996; Reed and Tanaka, 1996; Reed, 1997). Circular dichroism spectroscopy of taxol/Bcl-2 binding indicates that the drug induces a conformational change within a 10-16 amino acid long non-helical portion of the protein molecule, evidence that taxol primarily interacts with the flexible loop of Bcl-2 (Rodi et al., 1998).

The purpose of this work is to analyze similarities between sequences of phage-displayed dodecapeptides selected for taxol-affinity and sequences of proteins involved in apoptosis and mitosis; particularly those of tubulin and Bcl-2. These peptides have been selected from an extensive phage-displayed library of random peptides using modified affinity selection methods (Rodi et al., 1998). The binding properties of taxol-selected peptides are presumably determined by a

combination of their primary amino acid sequences and by the manner in which they are displayed on the surface of a phage particle. Similarities between the sequences of taxol-selected peptides and the sequences of proteins that bind taxol may therefore provide information about the way in which taxol binds to its molecular targets in terms of both relative importance of contact residues and conformational requirements of the ligand-binding site.

Binding of a small molecule drug to a protein involves (depending on the size of the ligand) 10-20 or more residues divided amongst several continuous stretches of amino acids, each only a few amino acids in length and separated by connecting peptides that are widely varying in length and make up the remainder of the protein. Within a typical discontinuous binding site not more than 5-7 amino acids in a row will interact with a small molecule ligand with molecular weight 300-1000 Daltons (Manon and Makowski, unpublished). Consequently, if similarities between the sequences of the peptides and Bcl-2 are based on their respective affinities for taxol, then it is unlikely that those similarities will be more than 5-6 amino acids in length. It follows that in order to identify sequence similarities that are reflective of ligand binding, any scheme for computing similarity scores must utilize a window of not more than 5-6 amino acids.

2. Materials and Methods

Screening of dodecapeptides for affinity to taxol was carried out as described elsewhere utilizing a commercially available phage-display library with random dodecamers displayed at the amino-terminal end of the pIII protein (New England Biolabs; Ph.D. system) (Rodi et al., 1998). Taxol was immobilized onto a streptavidin coated plate by using a derivative of taxol biotinylated at C-7 of the taxane ring. Previous SAR studies have shown that the anti-tumor activity of taxol is relatively insensitive to chemical substitution at this location (R.A. Holton, personal communication). Sequences of 69 taxol-affinity-selected peptides and 101 peptides randomly chosen from the library prior to screening were derived from the corresponding sequence of their viral DNA (Commonwealth Biotech, Inc.) using the manufacturers recommended primer.

The similarity between the 69 taxol-selected dodecapeptides (Rodi et al., 1998) and the sequence of Bcl-2 was calculated for all 234 of the hexapeptides along the 239 amino acid Bcl-2 sequence using the similarity matrix blossum62 (Henikoff and Henikoff, 1992). Very similar results were obtained using a similarity matrix derived from physical-chemical properties of the amino acids and developed specifically for this study. Those results are not reported here. Calculations of similarity score between the peptides and the protein were carried out either by using all peptides in all positions or using only those peptides that exhibited a high degree of similarity at a particular position (discarding all similarity scores below some selected minimum and, operationally, setting their similarity score to zero). The peptides isolated by biopanning for affinity to taxol presumably include high-affinity binders, relatively low-affinity binders, and other peptides that were isolated fortuitously due to non-specific attachment of the phage to the plate either through contact of the peptide or contact of other portions of the phage particle. Presumably, those present due to non-specific binding will not demonstrate significant similarity clustering to tested proteins. Therefore,

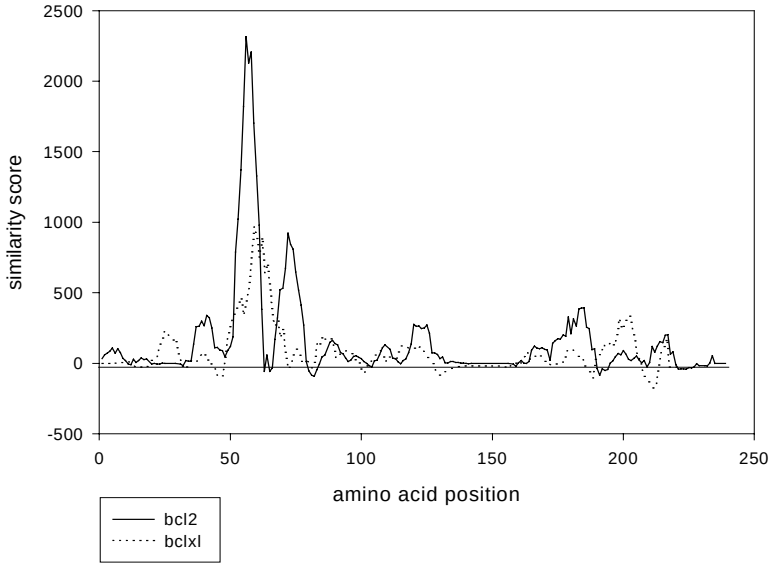

Figure 1 Similarity scores for the sequences of Bcl-2 and Bcl-X_L against the sequences of peptides selected for affinity to taxol computed using blossum62. The similarity scores of random peptides chosen without affinity selection have been subtracted from these scores to remove library bias. Significant homlogy is observed in the region of amino acids 40-80 in the flexible loop of Bcl-2. The similarity observed for Bcl-X_L in this region is significantly weaker.

setting a minimum similarity below which the peptide-protein segment comparison is ignored is an operational recognition that calculated similarity score below some fixed minimum threshold will be meaningless in any attempt to recognize similar binding properties of similar peptides. Experience reinforced that defining a minimum similarity score below which a peptide was ignored results in significantly better signal-to-noise ratios in the calculated similarity plots. This minimum was chosen to optimize the signal-to-noise ratio. For the Blossum62 matrix, the minimum was 18. This corresponds to at least 4 identities or similarities in the comparison of 6 amino acids in the peptide with 6 in the protein.

Since the phage-display library used for screening contains biases due to metabolic and structural constraints (Rodi and Makowski, 1997), the similarity between 101 prescreened dodecapeptides and Bcl-2 was also calculated for each hexapeptide along the Bcl-2 sequence, normalized for sample number and then subtracted from the similarity scores of the affinity-selected peptides.

Similar calculations were carried out for all known human α- and β-tubulins. Furthermore, peak similarity values were calculated for all other proteins identified by the search terms 'mitosis' (609 proteins) or 'apoptosis' (804 proteins) in an NCBI Entrez data base search of protein sequences.

Figure 2 Alignment of all peptides in which at least one hexapeptide exhibited a similarity score of 18 or more with the Bcl-2 sequence as computed using the Blossum62 similarity matrix. Identical or similar amino acids are indicated by underlined capital letters.

These calculations provide a base-line by which to evaluate the significance of similarities observed between the sequences of the affinity selected peptides and the sequences of Bcl-2-homologs or tubulins.

3. Results

Figure 1 shows a plot of the similarity scores calculated for Bcl-2 as a function of position along the Bcl-2 protein using the Blossum62 similarity matrix. The score plotted for any position reflects the average over that position and the next 5 amino acids. The similarity to Bcl-2 has a greater peak score and extends over a larger region than that of any of the other >1400 proteins tested. Figure 2 shows the sequences of taxol-selected peptides containing at least four identities or similarities to the Bcl-2 sequence aligned with the sequence of Bcl-2. Most of the highly homologous peptides exhibit similarity to sequences in the loop region (residues ~30-80). The region of highest similarity is broken into three shorter segments each involving ~10 amino acids, suggesting that the binding site for taxol may involve as many as 30 amino acids in the disordered loop either as contact or scaffolding elements. The positions of hydroxyls known to become phosphorylated in cells treated with taxol are indicated in this figure by (*), and are all either within or adjacent to the putative taxol-binding site. The position of pro59, identified as a hot spot for mutations that render Bcl-2 resistant to phosphorylation in taxol-treated cells, is indicated by (#). It

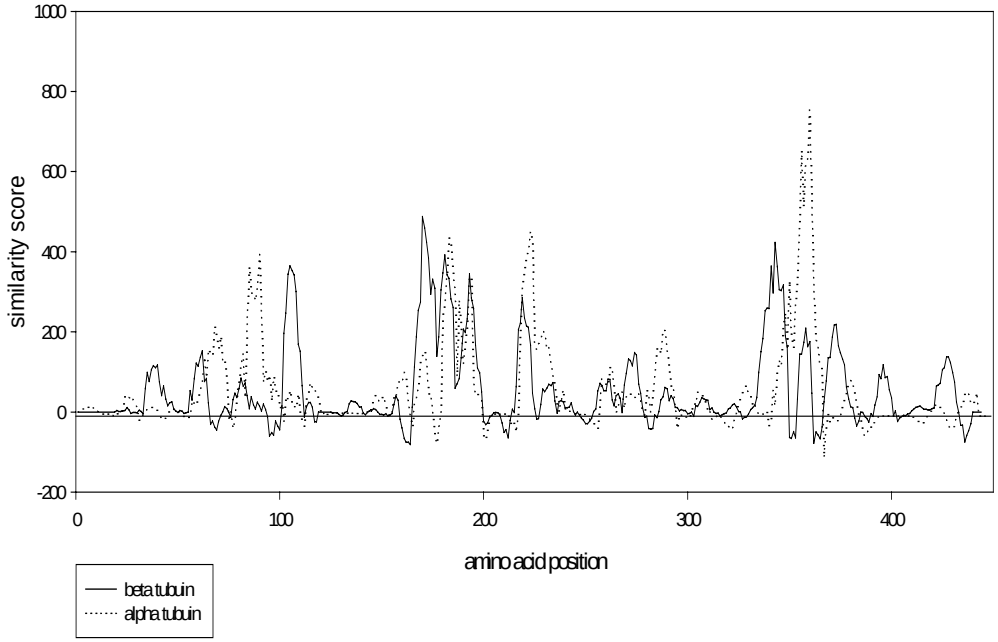

Figure 3 Similarity plots of taxol selected peptides with α- and β-tubulin calculated using the same procedure as in Figure 1. No regions exhibit the very high similarity score observed for the flexible loop of Blc-2. Few regions exhibit similarity scores higher than that observed for any protein chosen randomly from a data base, and the difference in the similarity curves for α- and β-tubulin does not appear to reflect the fact that taxol binds to β- but not α-tubulin.

falls within the region most similar to the sequences of the taxol-selected peptides. One phosphorlation site, ser87, is not in the loop region of the protein, but is an exposed residue on the surface of the molecule facing the loop (Muchmore, et al., 1996). There is weak similarity between the taxol-selected peptides and the region immediately surrounding ser87, which suggests that this region may also be involved in binding to taxol.

Figure 3 shows the results of similar calculations for α- and β-tubulin. The similarity score in the disordered loop region of Bcl-2 is significantly greater than it is for any part of either tubulin and many other proteins have regions of similarity to the selected peptides that are at least as great as those of the tubulins.. Nevertheless, it is instructive to consider the similarity plots in Figure 3 to determine if any substantive information about taxol-tubulin interactions may be derived, and to explore why the screen failed to detect similarities to taxol-binding regions of tubulin.

Three regions of contact have been identified between β-tubulin and taxol; residues 15-25; residues 212-222 and residue 275 (Nogales et al., 1998). Even on a relative scale, no similarity between the selected peptides and tubulin is detected in the regions of residues 15-25 or 212-222. The one region of relatively high similarity between the peptides and β-tubulin is in the region of residues 160-180 in the large beta5-helix5 loop which is close to the ribose ring of the bound nucleotide and apparently involved in nucleotide binding. This suggests that either taxol preferentially selects for peptides that tend to be in large, disordered loops (and that there are detectable similarities in the properties of all of these loops) or that some similarities exist between the peptides involved in binding of these small molecule ligands to proteins. The calculated similarity score in this region is in the same range as that calculated for the disordered loop of Bcl-X_L which exhibits relatively weak binding to taxol. Comparative analysis of the random peptides and the taxol-selected peptides in terms of overall flexibility (Bhaskaran and Ponnuswamy, 1988) indicates a preference by the taxol molecule for more flexible peptides.

4. Discussion

Disordered regions of proteins are known to be involved in binding to a broad range of different ligands (Spolar and Record, 1994; Dobson and Ptitsyn, 1997; Romero, et al., 1997; 1998; Dunker et al., 1998). We have shown here that there are detectable similarities between the sequences of phage displayed peptides selected for binding to taxol and the sequence of a disordered protein loop involved in taxol binding. Correlation of the similarity between the peptides and two similar anti-apoptotic proteins (Bcl-2 and Bcl-X_L) with the strength of their binding to taxol indicated that similarity was predictive for affinity in this case (Rodi et al., 1998).

Computed similarity scores for the taxol-selected peptides with either α- or β-tubulin were much lower than those for Bcl-2, and were not predictive for the specificity of taxol for β-tubulin over α-tubulin or for the position of taxol-binding in the zinc-induced tubulin sheets. The reason for this may be the very different scaffolds of the binding sites on tubulin compared with that for the peptides on the surface of the phage particles. The data reported here were generated using an unconstrained library in which the amino terminus of the displayed peptide corresponds to the terminus of the protein and is displayed at the edge of a large beta-sheet (Lubkowski et al., 1998). It is free to position itself anywhere with respect to the carrier protein or the rest of the peptide. This may provide a good representation of the form of the disordered loop of the Bcl-2 protein and its homologs. It is a poor representation of the taxol binding pocket of β-tubulin. It is possible that very different results would be obtained using a constrained library such as one constructed with a cysteine residue at each end of the random peptide. Usually the two cysteines are joined by a disulfide bond formed while the peptide is in the periplasm of the host bacterium. This bond forces the peptide into a loop and results in very different binding properties compared to the unconstrained library. Screening of constrained libraries may provide more information about the binding of ligands within pockets of relatively well-ordered protein domains.

The weak similarity identified between the taxol-selected peptides and a loop involved in nucleotide binding to tubulin suggests that loops involved in the binding of small molecule ligands

have similar properties. This is an experimental confirmation of the work of Romero et al. (1997) demonstrating that it is possible to identify disordered loops from amino acid sequences. It is also possible that the properties that result in an amino acid loop being disordered may provide it with properties that favor interaction with small molecule and other ligands.

The results presented here indicate that phage-displayed libraries represent a productive way to study the interaction of mobile protein loops with target molecules. Given our poor understanding of recognition processes involving disordered loops, this technique may provide significant information about the molecular recognition process.

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