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COMMUNICATIONS

Published on Web 03/14/2006

Synthetic Ligands for Apo-Neocarzinostatin

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Neocarzinostatin (NCS) is a 1:1 complex of an enediyne chromophore (NCSChrom), non-covalently bound to a 11 kDa protein (apoNCS).[1] It is derived from *Streptomyces carzinostaticus*[2] and has been used clinically as an anti-tumor antibiotic against a number of cancers.[3] It is believed that the primary role of the protein is to protect the highly labile chromophore from premature decomposition.[4] In addition, NCS exhibits selectivity toward certain cancerous tissues,[5] which has been exploited in the production of SMANCS, a conjugate of NCS and poly(styrene-*co*-maleic acid anhydride) that displays an improved tumor uptake and toxicological profile.[6]

We are exploring apoNCS as a generic protein system for sequestering small molecules for therapeutic applications. Recently, we have shown that attachment of the naphthoate portion of NCSChrom to a clinically used drug, Melphalan, confers on the drug an ability to bind apoNCS (K_d = 49 μM). This, in turn, improves the drug's stability with respect to hydrolysis.[7] From this work, it would appear that even ligands with relatively modest binding affinities (μM) can offer some useful advantage.

A prerequisite for the development of a more generic method for drug stabilization and delivery is the validation that apoNCS is able to bind non-cognate small-molecule ligands that may serve as generic anchors for drug molecules. A number of studies support the notion that wild-type apoNCS is able to bind non-cognate small molecules,[8,9] and in recent work, we describe a high-resolution structure of a ligand, which contains a fragment of the natural chromophore, bound to the apo-protein.[10] However, there is no high-resolution structural evidence to show that non-cognate entities can bind specifically to apoNCS. Here, we describe the first high-resolution structure of a completely non-cognate ligand bound to the protein. This provides unambiguous evidence that a new class of ligand can bind specifically to apoNCS. Furthermore, the mode of binding, although localized to the natural chromophores' binding site,[11] is different than that of the naphthoate based ligands, indicating the presence of multiple specific binding modes within the binding cleft of apoNCS.

Initial screening of a selection of small-molecule ligands (see Supporting Information) was carried out using established fluorescence and/or NMR (HSQC) assays.[12,13] This enabled the identification of the flavone motif as a new class of ligand for apoNCS. Indeed, α-naphthoflavone showed an affinity high enough (K_d = 12 μM) to encourage further evaluation.

To assess the location of the bound ligand, perturbations in the ^{1}H and ^{15}N chemical shifts of the protein were monitored and quantified upon ligand binding. This approach has been used to analyze protein−ligand interactions[14,15] by others and ourselves for the apoNCS system, where we have shown that the perturbations reflect the location of the ligand binding, revealed by high-resolution structures.[14] Using this approach, we determined that α-naphthoflavone and flavone **1** both bind specifically to apoNCS (see Supporting Information). To verify that a specific binding event was taking place, a high-resolution structure for the complex between apoNCS and **1** was determined. The ^{1}H, ^{13}C, and ^{15}N assignments for all residues within apoNCS were made as described previously[16,17] and have been deposited in the BioMagResBank (accession number 6889). These assignments were generally in good agreement with the values reported by others,[18−20] except the assignments for Ala28, Val34, Val40, Thr42, and Gly43 previously reported by us.[13] The solution structure of apoNCS was recalculated and refined using the program Cyana (version 1.1) with ^{13}C,^{15}N-labeled apoNCS. The coordinates for the final structures have been deposited in the RCSB Protein Data Bank (entry 2G0K).[21]

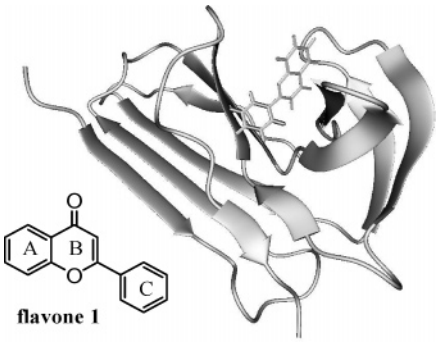

Figure 1. Ribbon representation of high-resolution NMR structure of flavone **1** bound to apoNCS (PDB entry 2G0L).

Since the solubility of flavone **1** is greater than that of α-naphthoflavone in aqueous systems, the solution structure of **1** complexed with the protein was solved by NMR. On the basis of the aforementioned assignments, sequence-specific ^{1}H and ^{15}N assignments were made for all residues, except for the proline residues at positions 3, 9, 49, and 105. The assignments have been deposited in the BioMagResBank (accession number 6888). The structure of the flavone complex, Figure 1, was calculated using the program Cyana and refined using the program HADDOCK (PDB entry 2G0L). An overlay of 58 structures showed that the structure was well defined, with an average rmsd of 0.67 ± 0.10 Å for backbone atoms and 1.00 ± 0.11 Å for heavy atoms. The mean structures of apoNCS and apoNCS−flavone complex were in good agreement, with an average rmsd of 0.51 ± 0.1 Å for backbone atoms and 0.99 ± 0.16 Å for backbone atoms in β-sheet regions.

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10.1021/ja058419i CCC: $33.50

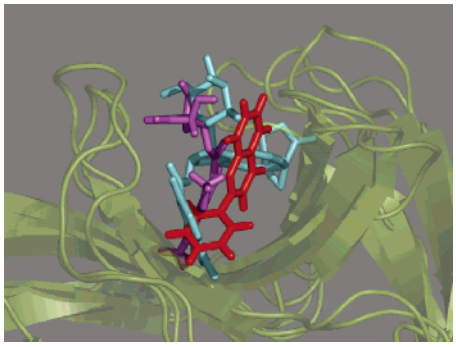

Figure 2. Overlay of the binding modes of three ligands for apo-Neocarzinostatin: NCS chromophore (cyan),[11] naphthoate analogue (purple),[13] and flavone **1** (red) bind in discreet and specific positions within apoNCS binding cleft (gray).

In holoNCS, the native chromophore is bound in a deep cleft formed by strands C1, C2, D, I, and J that is capped by loops L3, L7, and L9. In the present case, the orientation of **1** was unambiguously determined to be within a small hydrophobic patch of residues within the protein's binding cleft (Figure 1). It is clear that **1** binds toward the side of this cleft formed by loop L3 and strand J. The NMR structure was determined by just four unambiguous NOEs between the H3 of **1** and the amide protons of residues 35, 37, 76, and 96. The structure was refined using chemical shift perturbations as further restraints in HADDOCK. The resulting model indicated that flavone **1** binds toward the side of the cleft formed by loop L3 and strand J. The phenyl group (C) of flavone **1** is oriented toward the base of the cleft (strands I and J, in close proximity to residues 96 and 107) with the A−B ring portion of **1** positioned toward strand G and loop L7 (close to residues L77 and F78) at the entrance to the cleft. Flavone **1** is a much smaller molecule than the natural chromophore, and the loops L3 and L7 are repositioned accordingly. Loop L3 moves out from the cleft, with residues L45 and W39 shifting considerably, opening up the binding site; whereas loop L7 moves to close the top of the cleft with residue F78 moving in and so closing the back of the cleft. From this and our previous studies of synthetic chromophore binding using a naphthoate ligand (see Supporting Information),[13] it is clear that loops L3 and L7 can reposition to better accommodate ligands.

In the present case, flavone **1** bound with C7 and C8 of the flavone pointing toward the top of the binding site. Therefore, should a derivatized flavone bind in a similar way, the optimal place for conjugation of a drug entity would be at these positions. In addition, derivatives with substituents at the C2′ and C3′ positions on the rotatable phenyl ring appear to bind to apoNCS (data not shown). These positions could also point to the exterior of the binding site and, consequently, could be used for attachment of cargo. A comparison of the binding modes of NCSChrom, our previously reported naphthoate derivative,[13] and the flavone ligand **1** is presented and demonstrates the similar location but specific binding mode of each ligand (Figure 2).

In summary, we describe a new class of ligand for apoNCS based on the flavone moiety. This serves as a synthetically accessible and manipulable small molecule, which has potential as an anchor for the attachment of molecular cargo for binding to apoNCS. The ability of non-cognate chemical entities to bind apoNCS indicates that apoNCS has multiple binding modes for small-molecule ligands. It is interesting to note that other members of the family of chromoproteins Kedarcidin, C-1027, and Maduropeptin comprise proteins with considerable structural homology but which can accommodate structurally diverse ligands.[22] The present studies indicate that there are multiple, specific, and well-defined binding modes within apoNCS, and this offers very significant opportunities for binding small molecules for a variety of applications. These studies are encouraging for the development of protein−cargo complexes, which would have a variety of potential therapeutic applications.

Acknowledgment. We acknowledge BBSRC, EPSRC, GSK, Novartis, AstraZeneca, CEM, and Key Organics for financial support. We also acknowledge the MRC NIMR (Mill Hill) and the EPSRC Mass Spectrometry Service at Swansea.

Supporting Information Available: Details of ligand structures and HSQC analysis of perturbation of apoNCS by flavone and α-naphthoflavone. Pictorial representations of overlays of each of the three ligands for apoNCS. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA058419I