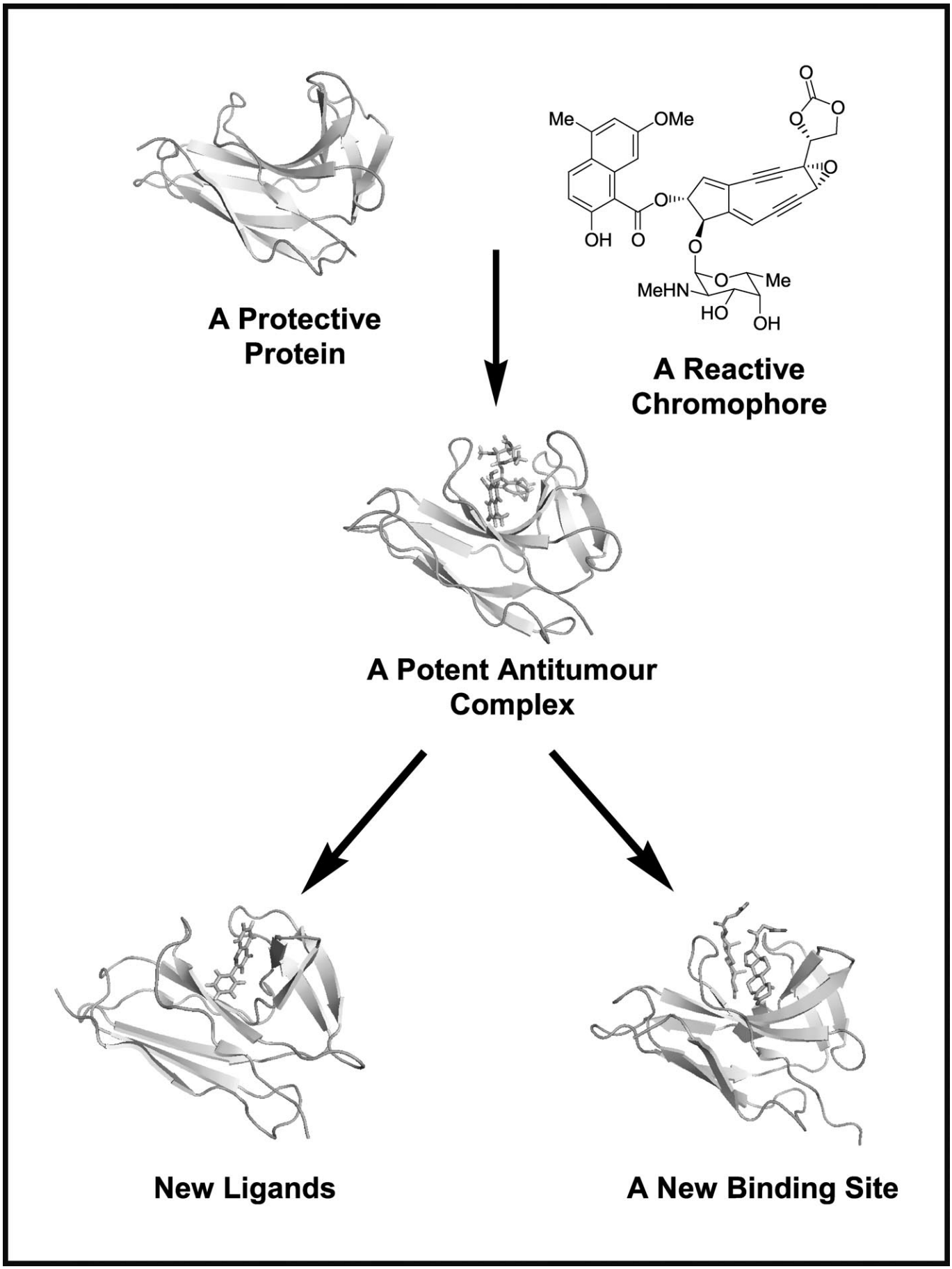

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Protein–Small Molecule Interactions in Neocarzinostatin, the Prototypical Enediyne Chromoprotein Antibiotic

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The enediyne chromoproteins are a class of potent antitumour antibiotics comprising a 1:1 complex of a protein and a noncovalently bound chromophore. The protein is required to protect and transport the highly labile chromophore, which acts as the cytotoxic component by reacting with DNA leading to strand cleavage. A derivative of the best-studied member of this class, neocarzinostatin (NCS), is currently in use as a chemotherapeutic in Japan. The application of the chromoproteins as therapeutics along with their unique mode of action has prompted widespread interest in this area. Notable developments include the discovery of non-natural ligands for the apoproteins and the observation that multiple binding modes are available for these ligands in the binding site. Mutation studies on the apoproteins have revealed much about their stability and variability, and the application of an in vitro evolution method has conferred new binding specificity for unrelated ligands. These investigations hold great promise for the application of the apoproteins for drug-delivery, transport and stabilisation systems.

1. Introduction

Interactions between proteins and small molecules are central to many biological processes, and provide targets for the majority of drug-discovery programmes. Usually, tight and specific binding depends on the formation of well-defined 3D protein structures that incorporate highly selective binding pockets. Our understanding of protein–small molecule interactions is increasing rapidly, particularly through the combination of chemical synthesis, protein mutagenesis and structural techniques such as X-ray crystallography and NMR spectroscopy. In turn, such studies are enabling the exploration of many systems in the search for new ligands for proteins and macromolecular complexes of interest in fundamental research and as potential leads for the pharmaceutical industry.

The enediyne chromoproteins represent an unusual example of protein–ligand interactions in that the protein serves to protect and transport a highly unstable small-molecule chromophore. These chromophores contain reactive enediyne functionalities and are potent cytotoxic compounds exerting their effect by reacting with DNA to cause strand cleavage. As a consequence, the chromoproteins are potent antibiotics, and highly effective antitumour agents. This review is not intended as an exhaustive overview of the large amount of research carried out on the enediyne chromoproteins. Rather, it will give an introduction to these intriguing complexes, and then discuss some of the reported studies on probing the apoproteins with new ligands and mutagenesis. Neocarzinostatin provides the central focus for the discussion as it is by far the most studied of the chromoproteins and is representative of the class.

2. The Enediyne Chromoproteins

In 1965 a proteinaceous substance, neocarzinostatin (NCS), was isolated from a culture of *Streptomyces carzinostaticus*,[1] and was found to possess broad-spectrum antibiotic activity. It was also discovered to exhibit antiproliferative activity against a number of tumour cell lines, both in vitro and in vivo.[2] Early biological studies were carried out before its true composition was known. It was not until 1979 that NCS was identified as a 1:1 noncovalent complex of a labile chromophore and a protein.[3] The chromophore was found to be responsible for the cytotoxic nature of this complex, although because of its instability to nucleophiles, heat, UV light and extremes of pH,[4] its structure remained elusive until 1985.[5] The key features of NCS chromophore are a core nine-membered

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enediyne ring, fused to a cyclopentene with pendent naphthoate and sugar groups (Figure 1 A). The 3D structure of the holocomplex was then reported by Rees and Myers in 1993 (Figure 1 B).[6]

2.1 The NCS chromophore and DNA damage

The biological activity of NCS derives from its ability to cleave DNA.[7] Its mechanism of action involves the naphthoate group

James Baker completed his MSci at the University of Nottingham in 2000. He then went to work as a research chemist at Novartis before commencing a PhD at the University of Bristol working with Prof. Kevin Booker-Milburn. His thesis considered the application of a photocycloaddition reaction to the total synthesis of stemoamide. He received his PhD in 2005 at which point he moved to UCL to carry out postdoctoral work with Prof. Stephen Caddick towards the total synthesis of the neocarzinostatin chromophore. He has recently been awarded an RCUK fellowship at UCL. His current interests include the development of novel synthetic methodology and the discovery of new ligands for proteins of biological significance.

Rhys Stoneman graduated with a BSc in Medicinal Chemistry and a DPhil in Chemistry from the University of Sussex under the supervision of Profs. Caddick and Woolfson. Following a postdoc position with Prof. Caddick at UCL, he worked for Dainippon Sumitomo Pharma Europe, then at Roche Products in the operational side of clinical research. He currently works for ICON Clinical Research as a Medical Writer.

Dek Woolfson gained a BA in Chemistry at the University of Oxford and a PhD in Chemistry at the University of Cambridge. He carried out postdoctoral research at the Universities of Cambridge, London and Berkeley (USA). He was appointed to Lecturerships in Biochemistry at the University of Bristol, and then at the University of Sussex where he was promoted to Professor of Biochemistry. In August 2005 he returned to the University of Bristol to take up a joint Chair in Chemistry and Biochemistry. His research interests have always been at the interface between chemistry and biology, with his main focus being on understanding sequence–structure relationships in proteins and applying this knowledge to rational protein design.

Mick Urbaniak received his D.Phil from the University of Sussex under the supervision of Profs. Caddick and Woolfson. For his postdoctoral work, he moved to the University of Dundee to study the parasite Trypanosoma brucei with Prof. Mike Ferguson, where he is currently a postdoctoral associate. He has continued to use an interdisciplinary approach to study galactose metabolism and GPI biosynthesis in T. brucei *and has developed assays for HTS of key* T. brucei *enzymes as drug targets in the Dundee Drug Discovery for Tropical Disease initiative. His current interests are in the application of chemical biology techniques to the study of parasite protein kinases.*

Fred Muskett received his PhD from the University of Edinburgh and then went on to complete a Masters degree in Biological NMR Spectroscopy at the University of Dundee. After five years of postdoctoral work in structural biology, he joined the MRC Biomedical NMR Centre at the National Institute for Medical Research (London, UK). In 2003, he joined the Henry Welcome Laboratories for Structural Biology (University of Leicester) as the NMR Centre manager. His current research interests are use of NMR methods to study protein–ligand interactions.

Stephen Caddick completed his BSc at Paisley College. After two years in the agrochemical industry he carried out his PhD at the University of Southampton and then postdoctoral work at Imperial College. In 1991 he joined Birkbeck College London and then moved to the University of Sussex in 1993, where he rose to the rank of full professor. In 2003 he joined University College London (UCL) as Vernon Professor of Organic Chemistry and Chemical Biology. His research interests include natural products synthesis and development of new methodologies for organic synthesis. Major areas of biological and biomedical research interests include heart failure, functional role of methylarginine processing enzymes, total and semi-synthesis of proteins, NMDA receptor–ligand interactions, drug design and delivery.

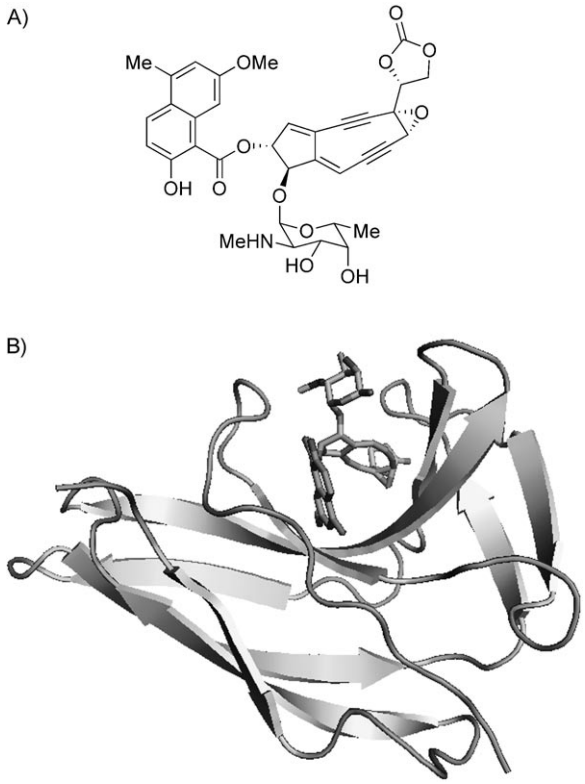
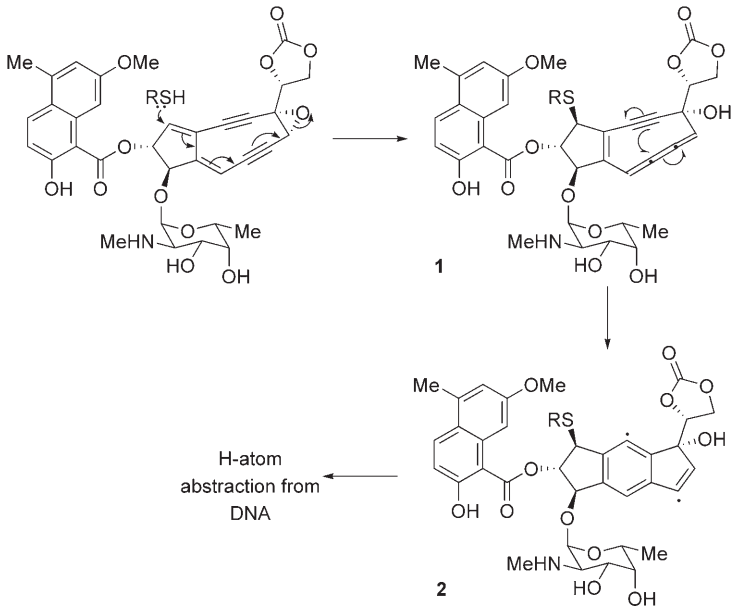


Figure 1. A) The NCS chromophore. B) HoloNCS is a 1:1 complex of a protein and the chromophore (PDB ID: 1NCO).

intercalating into DNA, positioning the rest of the molecule in the minor groove.[8,9] Activation occurs by nucleophilic attack of a thiol, most likely assisted by the basic nitrogen atom of the sugar moiety (Scheme 1).[10] There is some evidence that glutathione might function as this thiol in cells.[11] This attack leads to opening of the epoxide yielding an unstable cumulene species **1**, which has been observed at low temperatures by ^{1}H NMR spectroscopy.[12,13] Cycloaromatisation of this cumulene gives the diradical **2**, which abstracts hydrogen atoms from the deoxyribose sugar on DNA leading to its cleavage.[14]



Scheme 1. The mechanism of action of the NCS chromophore. Attack of a thiol group leads to cumulene formation and epoxide ring-opening. Cycloaromatisation then produces a diradical which abstracts H atoms from DNA leading to strand cleavage.

2.2 HoloNCS

NCS chromophore binds extremely strongly to apoNCS, with a generally quoted K_d of 0.1 nM.[15] From the crystal structure of the complex,[6] the two π faces of the enediyne ring of the NCS chromophore are sandwiched between Phe78 on one side and Phe52 and the disulfide bond of Cys37 and Cys47 on the other (Figure 2A). Phe78 is thought to play a key role in preventing nucleophilic attack at C-12, thus stabilising the chromophore.[6] The epoxide faces down into the hydrophobic pocket, which might protect it from acid-catalysed ring opening.[16] The naphthoate group is located at the bottom of the cleft and forms a number of H-bond interactions, for example its carbonyl group hydrogen bonds with the hydroxyl side chain of Ser98 and the methoxy group with the NH of Gly35 (Figure 2B).

The exact details of the delivery of the chromophore by the protein are still unclear. Lazarus et al. found that a complex of holoNCS bound to agarose, which is too big to enter cells, retains a significant amount of its inhibitory activity.[17] This suggests that the chromophore itself can enter the cell in the absence of the apoprotein. However, Maeda et al. have shown that fluorescently labelled NCS was observed to cross into the cytoplasm as well as the nuclei of human cells.[18] Myers et al. have also reported that fluorescently labelled NCS is readily transported into *S. cerevisiae*.[11] While it is thus clear that both the protein and the chromophore enter cells, it is not certain that they do so in the form of the holo complex. In other words, the exact point of dissociation is yet to be conclusively established. Interestingly, NCS has little apparent cytotoxic effect on unicellular eukaryotic organisms such as *S. cerevisiae*. In studying this resistance, Myers et al. found that the yeast rapidly overexpressed multiple DNA-damage repair genes during NCS exposure, and they also identified a pathway by which NCS protein is exported from the cell.[11]

2.3 SMANCS

The potency of NCS as an antitumour agent surpasses that of the widely used antitumour agents 5-fluorouracil and cisplatin at their minimum effective concentrations.[19] NCS can inhibit tumour cell growth in the nanomolar range, whereas many low molecular weight compounds do so in the micromolar range.[2] The stumbling block for using NCS clinically, however, is its severe toxicity.[20] Furthermore, it only has an in vivo half-life of ~1.9 min resulting in the need for carefully controlled infusion for clinical usage. These

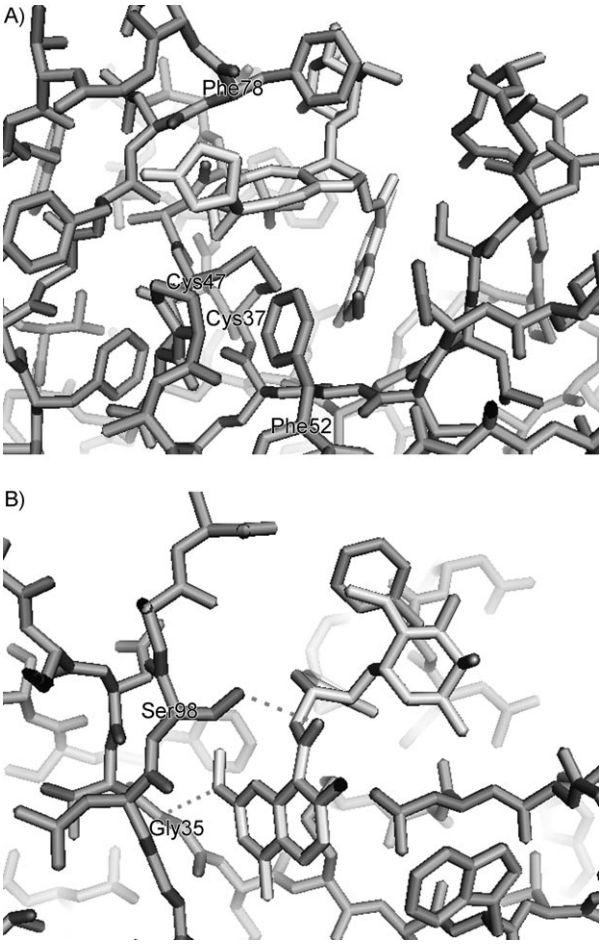


Figure 2. A) NCS chromophore in its binding site (PDB ID: 1NCO). The chromophore is sandwiched between Phe78 on one side and Phe52 and the disulfide bond of Cys37 and Cys47 on the other. B) The naphthoate is located at the bottom of the cleft, and forms H bonds with the residues Ser98 and Gly35.

limitations have been partially overcome by the synthesis of a poly(styrene-co-maleic acid/anhydride) (SMA) conjugate of NCS, thus known as SMANCS. The polymer is linked to the amino groups of the protein at Ala1 (the N terminus) and Lys20. SMANCS has an in-vivo half life ten times that of NCS, and also has the advantage that as a macromolecule it is retained more by solid tumours than in normal organ or tissue.[19] This improved stability and selectivity has led to the clinical approval of SMANCS as an antitumour agent with impressive application to date.[21] For example in the case of hepatomas it has been shown to reduce the size of tumours in 90% of patients.[19]

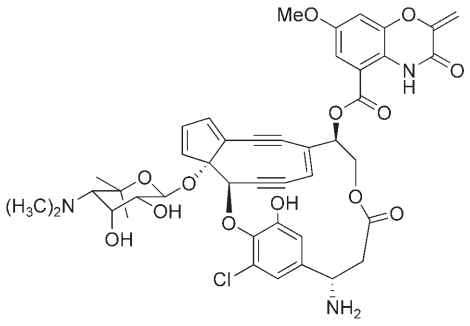
2.4. Related enediyne natural products

Since the discovery of NCS a number of related species have been isolated. Dynemicin,[22] calicheamicin[23] and esperamicin[24] represent a related class of compounds, which have ten-membered ring enediynes and lack any associated protein. These compounds are considered not to require the stabilisation offered by the protein because of the reduced strain, and therefore the reduced reactivity, of the larger enediyne ring. They are, however, still potent antitumour antibiotics, with the calicheamicin immunoconjugate known as mylotarg showing promising results for the treatment of leukaemia.[25–27] N-1999A2 is the only enediyne with a nine-membered ring that has been isolated that does not have a protective protein associated with it.[28]

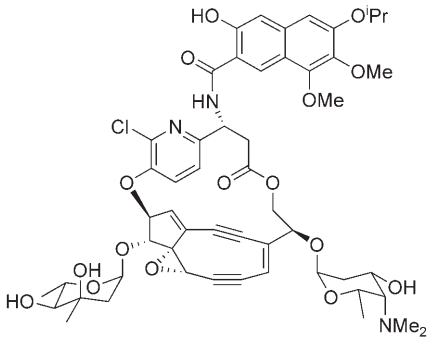
Interestingly, it has recently been reported that the nine- and ten-membered ring enediynes share a common polyketide biosynthesis pathway.[29–31]

2.5 The enediyne chromoproteins

The enediyne chromoproteins can be roughly divided into a homologous group containing neocarzinostatin, kedarcidin, C-1027, actinoxanthin and macromomycin (otherwise known as auromomycin); and other nonhomologous examples including maduropeptin, largomycin and sporamycin. Very little has been reported on the structural details of the nonhomologous enediyne chromoproteins. The homologous chromoproteins have chromophores containing nine-membered ring enediynes, bound to proteins that are all ~11 kDa in size. In the case of macromomycin and actinoxanthin the chromophores have so far proved too labile to allow structure determinations. As with NCS, the structures for C-1027[32] and kedarcidin[33] chromophores have been reported.



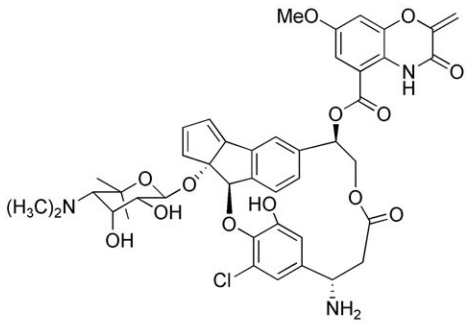
C-1027 chromophore



kedarcidin chromophore

In the absence of its stabilising protein C-1027 chromophore is the most reactive member of the class. It does not require activation in order to cyclise[34–37] and is in fact in equilibrium

with its cycloaromatised diradical while in the protein site.[36] This poses another question, namely, why is the chromophore not deactivated by abstracting hydrogen atoms from the protein? Tanaka et al. propose that deactivation is prevented by the lack of abstractable hydrogen atoms in the proximity of the radicals in the complex. To examine this they obtained a structure of the aromatised product bound to the apoprotein (Figure 3).[38]



Aromatized C-1027 chromophore

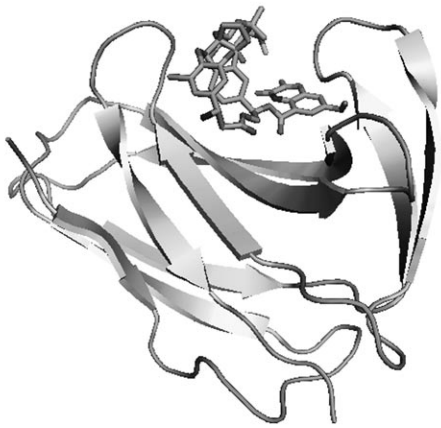


Figure 3. The aromatised chromophore of C-1027 and the aromatised chromophore of C-1027 bound to apoC-1027 (PDB ID: 1HZL).

The aromatised chromophore is an excellent mimic for the key diradical intermediate, with just two extra hydrogen atoms on the benzene ring. The structure revealed Gly96 and Pro76 as the closest residues from which hydrogen atoms could be abstracted, and that the only appropriate hydrogen atoms would be orthogonal to the diradical, thus preventing abstraction (Figure 4).

A solution structure of the C-1027 cycloaromatised chromophore/DNA heptamer complex has also been determined and reveals that the benzoxazine intercalates between DNA base pairs and that the aminosugar binds in the minor groove through H-bonding.[39] The aminosugar is well exposed to the solvent as is part of the benzoxazine. Tanaka et al. propose that holoC-1027 might therefore enter the cell, coming sufficiently close to the target DNA to allow the sugar moiety to interact with it, which is then followed by intercalation of the benzoxazine and transfer of the whole chromophore.[38] This

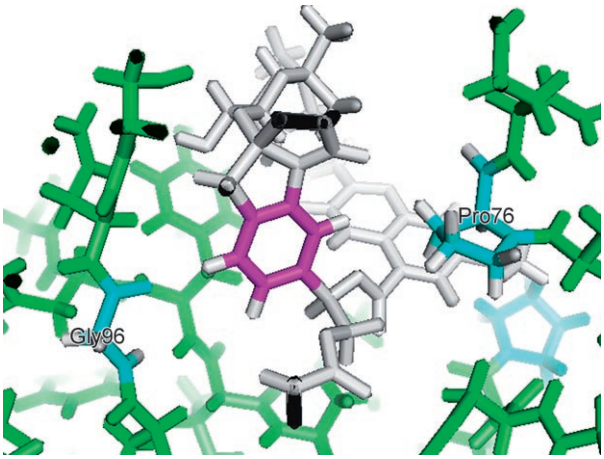


Figure 4. The aromatised chromophore bound to apoC-1027 (PDB ID: 1HZL) clipped to show the benzene (magenta) with the closest residues Gly96 and Pro76 (both cyan) also highlighted. The protein is shown in green, with the rest of the chromophore in white.

would avoid the highly reactive chromophore from being quenched by abstracting hydrogen atoms from other sources.

2.6 The apoproteins

All of the homologous chromoproteins have had their apoprotein structures determined.[40–43] The apoproteins themselves are all very similar, they are 108–114 residues long, include a high proportion of hydrophobic residues and a highly conserved tertiary structure (Figure 5). They all have seven β-strands arranged in a layered structure of three β-sheets; though the third sheet is rather small. This results in clearly defined cavities comprising mostly hydrophobic residues for chromophore binding. The structures are all crosslinked with two disulfide bonds. Nonetheless, there are interesting differences between the chromoproteins. The binding sites for NCS and macromomycin are deeper and narrower than those of the other three. The differences are illustrated by the discovery that each protein only binds its corresponding chromophore.[44]

An intriguing possibility suggested by a number of groups is that the proteins not only serve to transport and protect the reactive chromophore, but are also biologically active themselves; in particular they have been proposed to act as proteases.[45] It has been suggested that histones, which are involved in forming nucleosomes with DNA, might be the target of these proteases thus providing the chromophore with easier access to the DNA target.[46] However, Heyd et al. have reinvestigated the purported proteolytic activity of NCS. They used recombinant apoNCS produced by expression of a synthetic gene in *E. coli*. Full characterisation confirmed that the protein was identical to the natural apoNCS. The recombinant and natural proteins differed only in their proteolytic activities, with the former showing weaker activity. Furthermore, the authors carried out ion-exchange chromatography on apoNCS and found a significant reduction of the proteolytic activity; this suggests that the proteolytically active component was

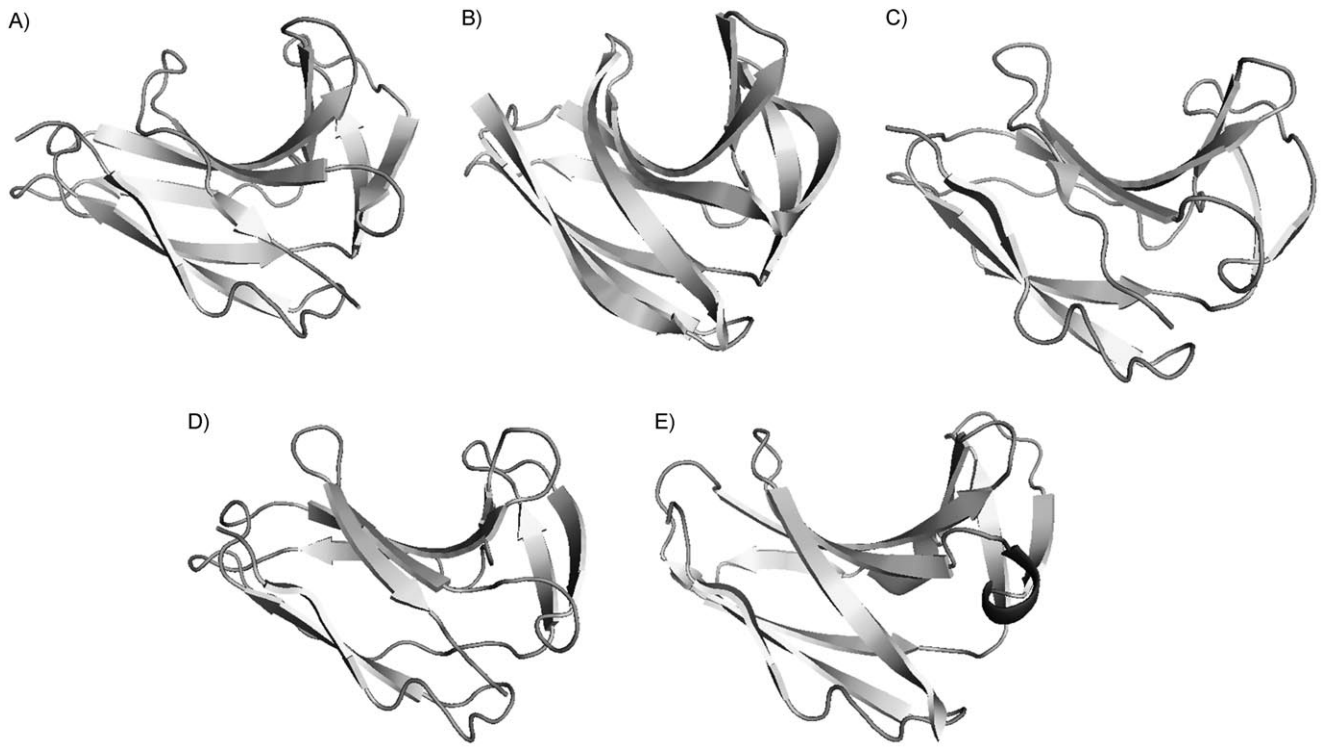


Figure 5. The structures of the apoproteins taken from the PDB. A) neocarzinostatin (1NOA), B) macromomycin (2MCM), C) actinoxanthin (1ACX), D) C-1027 (1J48) and E) kedarcidin (1AKP).

being removed by purification. They conclude that the proteolytic activity is in fact caused by a minor contaminant present with apoNCS. This was further confirmed by the use of anti-NCS antibodies, which made it possible to remove any apoNCS, leaving just the impurities that retained a similar level of protease activity. Our work has also confirmed that purification of apoNCS leads to loss of proteolytic activity.[47] It is clear that apoNCS does not behave as a protease, while this has yet to be proven in the cases of all the chromoproteins, it is likely that this is a general result.

3. Probing the Chromoproteins with Small Molecules

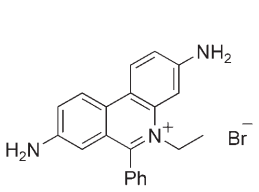
The functional role of the apoproteins in this class is to bind, protect and possibly transport reactive small molecules to their target site, DNA. This makes them potential candidates for developing new drug-delivery systems for other chemotherapeutics. In order to assess this potential, small-molecule ligands other than the chromophores themselves have been tested for binding and complex formation. Understanding how any new ligands bind to the apoproteins then helps to identify the key interactions involved. The potential also exists to identify strong binding motifs to which other drug molecules can then be attached. Before discussing discoveries in this area it is worth mentioning the key analytical tools that have been used to probe the binding of ligands to the chromoproteins.

3.1 Analytical methods

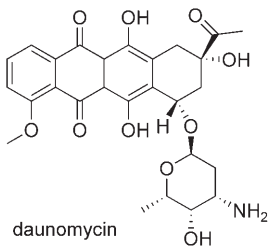
The most common methods that are used to determine the binding strength of a ligand to a protein are isothermal titration calorimetry (ITC) and florescence titration.[48] ITC measures the heat change when the ligand is added to a solution containing the protein. From this the enthalpy (ΔH) of the interaction can be determined as can the binding affinity (K_a). Fluorescence on the other hand requires that either the ligand or certain residues in the protein (such as tryptophan) fluoresce and that a change in the fluorescence is observed on binding. In the case of the chromoproteins the chromophores are conveniently fluorescent, leading to this method being favoured. The location at which the ligand is bound in the protein can then be determined by using crystallographic or NMR spectroscopic techniques.[49–51] The key to using NMR to determine ligand binding is the ability to assign both the protein's and the ligand's individual NMR spectra. Once this has been done, a convenient method for estimating ligand location is then by simply observing changes in the chemical shifts of peaks on binding, which are most likely to occur at the binding interface.[47,52]

3.2 Non-natural binders of the chromoproteins

Ethidium bromide (EtBr) is a fluorescent compound that intercalates in DNA and in so doing its fluorescence increases by almost 20-fold. It has thus become widely used as a stain for nucleic acids. In common with many DNA intercalators it has a



ethidium bromide (EtBr)



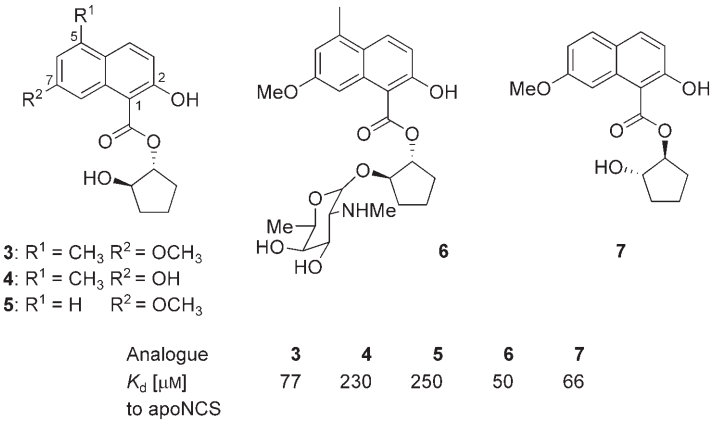
daunomycin

polyaromatic structure. In this respect, it bares some similarity to the naphthoate moiety of NCS chromophore. Indeed, EtBr binds to apoNCS with a K_d of approximately 1 μM as established by fluorescence.[53] Further evidence of binding was initially obtained by a simple ^{1}H NMR in D_2O. In this case two methyl proton peaks assigned to Leu45 of apoNCS were identifiable at the high-field end of the spectrum at −0.14 and −0.24 ppm and located in the binding cleft. They were observed to move significantly to −0.78 and −0.87 ppm on addition of EtBr. A further indication of binding was the strong perturbation in the aromatic protons of the EtBr itself. These are good examples of how a simple NMR analysis can confirm binding. At the time of this research the protein had not been cloned and thus isotopic labelling of the protein was not viable, and the complex was assigned by using homonuclear methods.[53] NOESY experiments located the EtBr in the same cleft in which the chromophore locates. Furthermore it was proposed that the EtBr was binding in the same vicinity of the cleft in which the naphthoate and enediyne are located in the natural system. Unfortunately, the complex has not been placed in the PDB (Protein Data Bank) for analysis. Nonetheless, EtBr has become an extremely convenient tool for measuring the functional properties of NCS and its variants. This is particularly useful considering the instability of the natural chromophore.

The antitumour agent daunomycin has also been found to exhibit an affinity for apoNCS.[54] This drug and NCS chromophore share more similar functional groups with sugar moieties in addition to the planar aromatic motifs. Once again, NMR was used to confirm that binding takes place in the natural binding cleft.

3.3 Analogues of the NCS chromophore

Significant practical difficulties are associated with the design and synthesis of analogues of the NCS chromophore to probe the structure–activity relationships. The synthesis of the NCS chromophore itself highlights this point, as much synthetic work has been carried out in the area culminating in only two successful total syntheses to date.[55,56] The main problem is the inherent instability of the chromophore's core nine-membered enediyne structure. An interesting experiment was carried out by Edo et al. in which a 30-fold excess of β-naphthol was added to a solution of the holoNCS, causing the complete release of the chromophore. In contrast, a 5000-fold excess of D-galactosamine was required to achieve this. The suggestion is that the naphthoate is the key component of chromophore binding, which has been borne out subsequently. Hirama et al. have synthesised various naphthoate-based analogues and measured their binding to the apoprotein by fluorescence titration.[57] They find that the simple analogues **3**–**7** all bind apoNCS moderately (Scheme 2). There is virtually no increase going from alcohol **3** to glycoside **6** suggesting that the sugar



3: $R^1 = CH_3$ $R^2 = OCH_3$
4: $R^1 = CH_3$ $R^2 = OH$
5: $R^1 = H$ $R^2 = OCH_3$

6 **7**

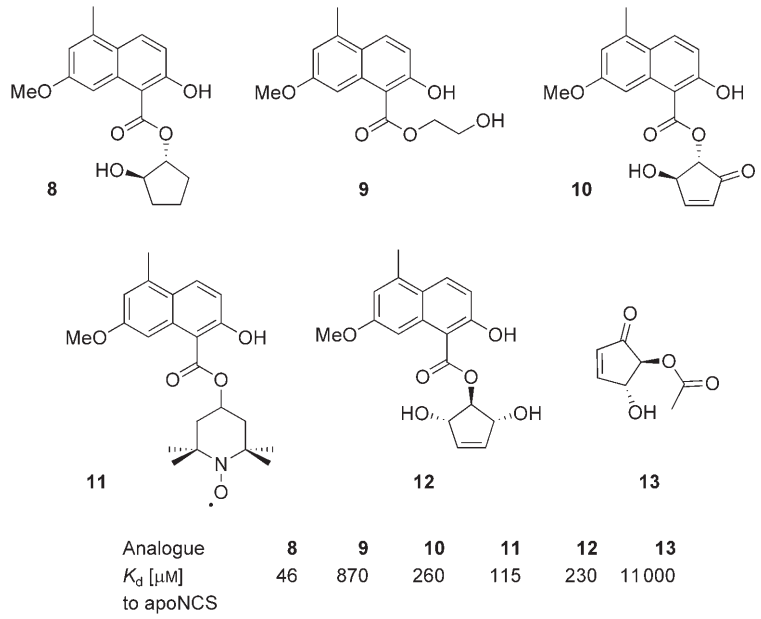
Analogue	**3**	**4**	**5**	**6**	**7**
K_d [μM] to apoNCS	77	230	250	50	66

Scheme 2. The binding of simple naphthoate analogues to apoNCS.

does not play such a significant role in binding to the protein. The loss of the C-5 methyl or the methyl of the methoxy group at C-7 led to a reduced binding affinity, proposed to be due to the loss of van der Waals interactions with Gln94 and a CH–π interaction with Phe52, respectively. The opposite enantiomer **7** showed no reduction in affinity.

We have also investigated the ability of apoNCS to accommodate molecules other than the natural chromophore in its binding site. To do this, we synthesised a number of naphthoate esters and investigated their binding to recombinant apoNCS (Scheme 3).[47]

Florescence titration methods were used to determine the dissociation constants for the apoNCS–small molecule complexes. As shown in Scheme 3, these naphthoate analogues bind moderately to the apoprotein. We then used an alternative method to attempt to determine the location that these analogues might occupy in the binding site. The use of spin-labelled ligand **11** allows the residues of the protein that are in its proximity to be identified by increased line widths in NMR spectra. This effect was observed for residues in the apoNCS binding site, but unfortunately the chemical shift perturbation caused by the naphthoate was sufficient to hinder assignment in crowded regions of the spectra. Instead the solution structure of the apoNCS–**12** complex was determined by a full NMR investigation (Figure 6).[47] We found that **12** does indeed sit in the binding cleft of apoNCS, but that it has an altered orientation with respect to the natural chromophore. The naphthoate portion adopts a more central position and has rotated, losing the hydrogen bond with the backbone NH of Gly35 seen in the natural complex. The hydrogen bond between the carbonyl group and Ser98 has also been lost, but a new one between this residue and the hydroxyl group of the naphthoate has



Analogue	8	9	10	11	12	13
K_d [μM] to apoNCS	46	870	260	115	230	11 000

Scheme 3. The binding of naphthoate and cyclopentenyl analogues to apoNCS.

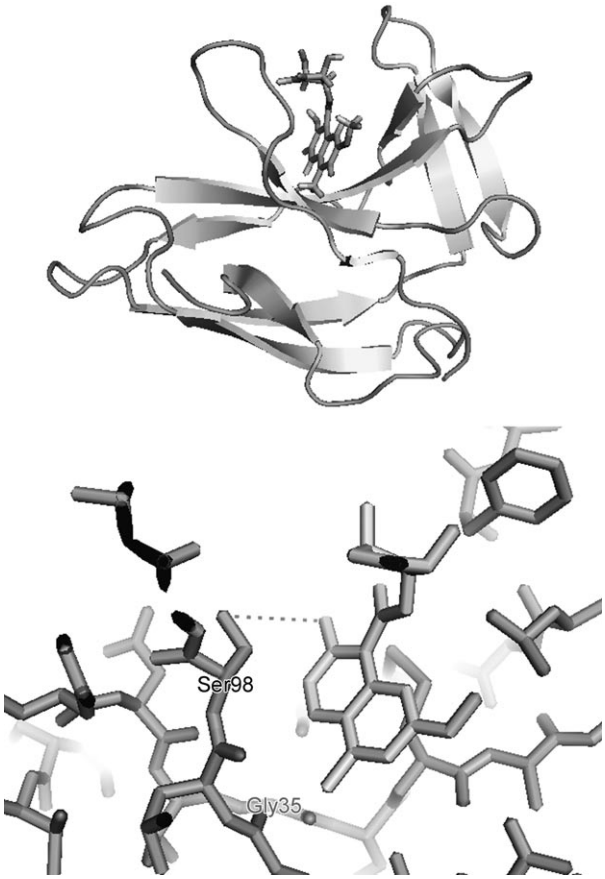


Figure 6. The binding of analogue **12** to apoNCS (PDB ID: 1J5I). The naphthoate portion adopts a more central position in the binding cleft, and has rotated forming an H-bond with Ser98 and losing the H-bond with Gly35.

been established. These discrepancies in the binding of ligands to proteins highlight the importance of detailed structural studies as a basis for rational small-molecule ligand design.

3.4 Flavones as new binders of apoNCS

With apoNCS revealed to bind a number of small aromatic molecules, we decided to carry out a screen for completely new ligands. The long-term aim of this work is to explore hits as anchors in a generic drug-delivery system. The screen identified the flavone motif **14** as new class of binder.[58] Specifically, α-naphthoflavone **15** showed an affinity high enough ($K_d = 12$ μM) to encourage further evaluation. To verify specific binding, an NMR structure of **14** complexed with apoNCS was determined (Figure 7).

In the case of **14**, C-7 and C-8 of the flavone point toward the top of the binding site. Assuming a derivatised flavone bound in the same way these would therefore be the optimised positions for conjugation of a drug entity. In addition derivatives with substituents at the C-2′ and C-3′ positions on the rotable phenyl ring appear to bind to apoNCS. These positions could also point to the exterior of the binding site and could also be sites of attachment. The location of **14** was determined to be adjacent to a small

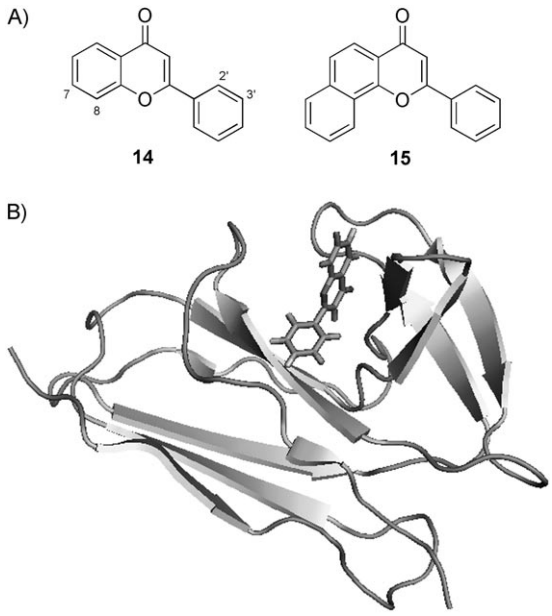


Figure 7. A) Flavone **14** and α-naphthoflavone **15** bind to apoNCS. B) Flavone **14** bound to apoNCS (PDB ID: 2G0L).

hydrophobic patch of residues within the protein's binding cleft (Figure 8). The phenyl ring is located at the base of the cleft, in a pocket formed by Leu45, Gly96 and Gly107. An H-bond interaction is observable between the flavone carbonyl group and the amide NH of Cys47.

The orientation of the flavone is very different to the naphthoate-based ligands including the NCS chromophore itself. It is clear therefore that apoNCS has multiple binding modes for small-molecule ligands. This can be seen clearly when the binding of the three ligands are overlaid (Figure 9).

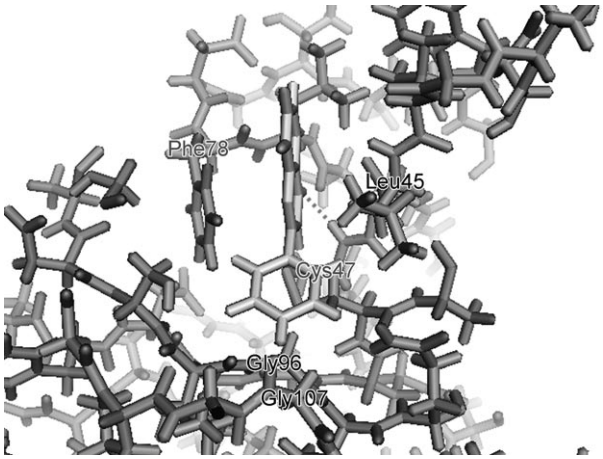


Figure 8. The binding of flavone **14** to apoNCS (PDB ID: 2G0L). The phenyl ring is located at the base of the cleft in a pocket formed by Leu45, Gly96 and Gly107 and an H-bond interaction is observable between the flavone carbonyl group and the amide NH of Cys47. Phe78 has moved into the cleft.

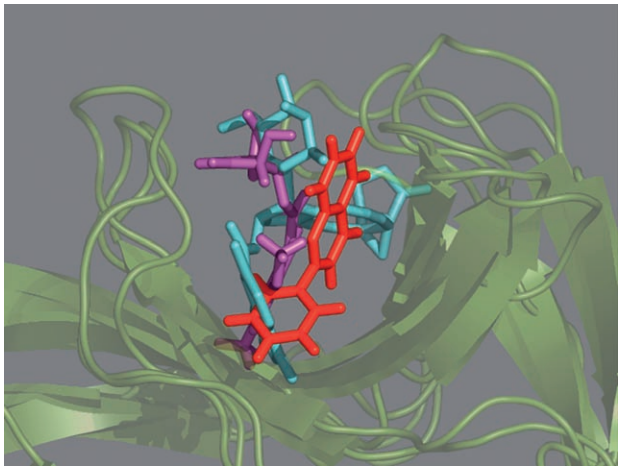


Figure 9. Overlay of the bound conformations of NCS chromophore (cyan), naphthoate analogue **12** (purple) and flavone **14** (red).[58]

It is also interesting to note the variations in the protein structure with different ligands bound. Particularly notable is the movement of the loop containing Phe78 on the binding of flavone, which essentially places this residue into the binding cleft (Figure 10).

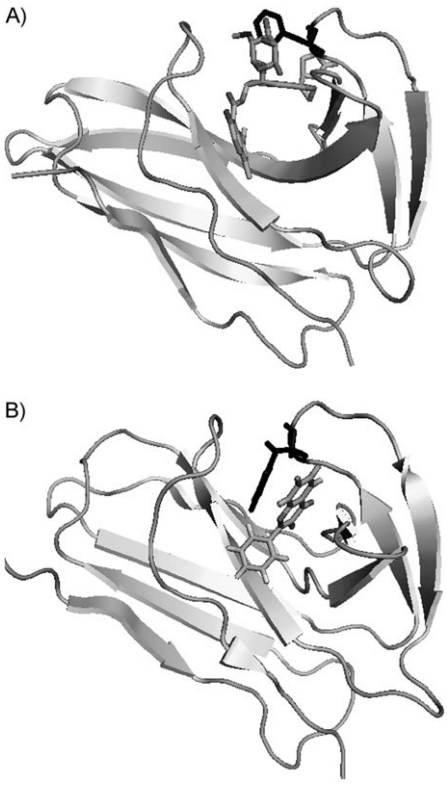
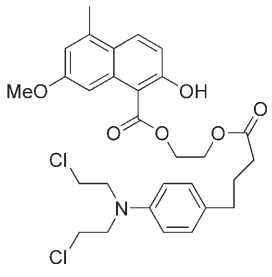


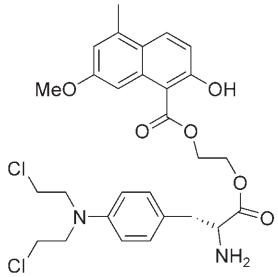
Figure 10. ApoNCS A) with the chromophore (PDB ID: 1NCO) and B) with flavone (PDB ID: 2GOL). Phe78 is shown in black in each case.

4. Ligands as Anchors: Towards a Drug-Delivery System

The use of apoNCS as a stabiliser and transporter for other reactive small molecules holds much promise. We have considered using the naphthoate group—which, as discussed, plays a key role in binding the natural chromophore to the protein—as an anchor for the attachment of other drug molecules.[59] The nitrogen mustards chlorambucil and melphalan represented ideal candidates as drugs to attach. They are both widely used chemotherapeutics, acting by alkylating DNA and producing interstrand crosslinking.[60] The efficiency of these agents, however, is limited by their deactivation through reaction with nucleophiles such as water, thiols and proteins, and further through their low affinity to DNA. The prospect of linking them to the naphthoate group and then allowing them to form complexes with the protein was aimed at addressing both these limitations. The naphthoate group as a DNA intercalator would strengthen the overall interaction with DNA, while the protein would protect the molecule from attack. Chlorambucil and melphalan were thus coupled to the naphthoate by utilising ethylene glycol as a short, flexible linker. The chlorambucil conjugate **16** and the melphalan conjugate **17** were then tested for their biological activity and their binding to apoNCS.



16



17

The in vitro cytotoxic activity of these compounds against a human leukaemia cell line[61] was then measured (Table 1). Conjugation to the naphthoate increased the potency of melphalan, but not that of the chlorambucil. This is consistent with previous work that suggested that a positively charged side chain is required for intercalation of naphthalene derivatives.[62] This also reasserts the potential importance of the positively charged aminosugar in the NCS chromophore in binding to DNA. The ability of the naphthoate conjugates to bind to apoNCS was measured through fluorescence-quenching titration. The binding of the chlorambucil derivative **16** was weak and did not allow an accurate dissociation binding constant to be determined. In contrast the melphalan derivative **17**, which is one methyl group shorter, showed significant binding. For reference, the nitrogen mustards themselves were tested for binding to apoNCS, and negligible change in fluorescence suggested that no binding took place.

Table 1. Measurements of the cytotoxicity and binding affinity to apoNCS of melphalan, chlorambucil and their naphthoate conjugates.

Compounds	IC_{50} [μM]	K_d (ApoNCS)
melphalan	30 ± 10	–
17	7 ± 4	47 ± 5 μM
chlorambucil	95 ± 25	–
16	200 ± 50	> 1 mM

The next test was to determine whether the protein protected the reactive substrate. Reversed-phase high-performance liquid chromatography was used to follow the hydrolysis of **17** in the presence and absence of apoNCS. Indeed, the half-life of **17** increased from 3.5 ± 0.5 to 8.1 ± 1.5 h in the presence of one equivalent of apoNCS. In contrast, in the case of the chlorambucil derivative **16** no effect was observed, reflecting its poor binding to the protein. Thus, apoNCS can act to protect non-natural ligands from attack by the solvent, producing a measurable effect on the lifetime of the small molecule even when only moderate binding occurs. The final question was then whether the complexation of the melphalan derivative **17** with apoNCS would increase its in vitro cytotoxicity. We found no improvement on the IC_{50} against a human leukaemia cell line in vitro. This is consistent with previous results on NCS, which show that while the holo complex is essential for in vivo activity the chromophore alone is as active as the holo complex in vitro.[4,63]

These results illustrate the potential for harnessing apoNCS as a more generic drug delivery system. The naphthoate–melphalan conjugate bound only moderately to the apoprotein and an observable stabilisation resulted. An improvement of the K_d for such a complex could be expected to further improve this effect. Protein engineering offers the potential to alter the binding site of such a protein, and might offer the prospect of tailoring the site to bind such a conjugate more strongly.

5. The Stability of the Apoproteins

The apoproteins must be resistant to unfolding prior to delivering their chromophores as this would result in the loss of function. Indeed it has been found that the T_m of apoNCS far exceeds ambient temperatures over the broad range of pH 4–10.[64] In a complex physiological environment, however, there are many other conditions that apoNCS must be capable of surviving, if it is going to successfully deliver the "warhead" to the DNA. Initial research by Maeda et al. found that apoNCS was extremely resistant to reduction of its two disulfide bonds, even in the presence of the highest concentrations of urea or guanidine hydrochloride.[65] The hydrophobic cellular membrane is a challenging environment in which NCS must remain folded, assuming it is required to deliver the chromophore into the cell. In this vein, Chin and Sundhahar explored the stability of apoNCS to a range of chemical denaturants.[66] They investigated the effect of organic solvents, methanol, ethanol, isopropanol, *tert*-butyl alcohol and acetonitrile on the conformational stability of apoNCS. ApoNCS is considerably more stable to organic solvents than proteins of a similar size, such as cytochrome *c* and hen-egg-white lysozyme. They also considered the detergent SDS, which is a loose mimic of the lipid molecules of cell membranes, and is 500–1000 times more effective than urea and guanidine hydrochloride at denaturing proteins.[67] No change in apoNCS conformation was observed up to 887 mM concentrations of SDS, which is the highest concentration possible to monitor by CD spectroscopy. These results confirm that apoNCS is a robust protein and is thus likely to be a stable vehicle for drug delivery.

6. Mutation Studies

Mutations have been made to the apoproteins to probe the chromoprotein systems. Hariharan et al. have used mutation studies to investigate the mechanism by which the ligand is released from the protein.[68] That the crystal structures of apoNCS and holoNCS are almost superimposable[6] suggests distinct structural changes might not be required for the binding or release of NCS chromophore. The authors therefore speculate that the movement of specific side chains might control the release of the chromophore.[68] Furthermore, the release of the noncovalently bound ligand might be triggered by an agent within the cell, and thus the key residue is likely to be at the exposed surface of the protein. It is also likely to be in a crucial position for opening and closing the binding cleft. Mutation studies were thus carried out on a few key residues, then the release of NCS chromophore from each of the mutant holo complexes was measured by fluorescence. Conveniently, glutathione (GSH) was found to form an adduct with any free chromophore that was 60 times more fluorescent than the chromophore itself. Thus, increases in fluorescence could be used to measure the release rate. Very little change was noted with most of the mutants, with the exception of those in which Phe78 was mutated, in which case a significant increase in the release rate was observed. This suggests that Phe78 is potentially a key residue involved in the release of

the ligand. Comparing the structures of holoNCS with apoNCS it is clear that this residue has moved notably (Figure 11). In the holo complex Phe78 bridges across the cleft, apparently

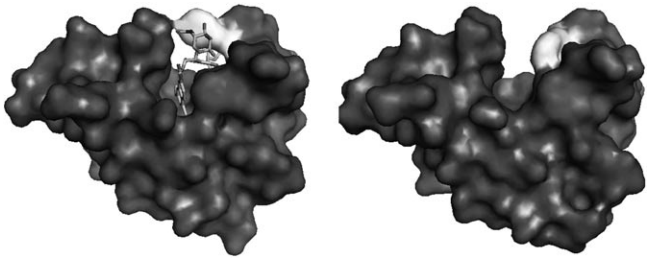


Figure 11. The surface structures of holoNCS (1NCO) and apoNCS (1NOA). The protein is shown in dark grey with the residue Phe78 in white and the chromophore in stick representation.

holding the chromophore in place, whereas in the apo form it has moved into the wall of the protein opening up the cleft. An alternative explanation for the increased rate of GSH adduct formation in the Phe78 mutants is due to the key role this residue plays in shielding the chromophore from attack.[6]

6.1 In vitro evolution

An in vitro evolution of NCS has been carried out by Minard et al. with the aim of creating a new binding site for a target molecule unrelated to the natural ligand.[69] Testosterone was chosen as this molecule because the structure of testosterone–protein complexes are known for comparison and also because analogues of testosterone are available for selectivity studies. Thus libraries of around 1×10^9 independent clones were created in which up to 13 of the side chains pointing toward the binding cleft were mutated to multiple residues simultaneously. This was done by replacing regions of NCS with synthetic cassettes encoding mutations at the defined positions. Phage display was then used to select the variants that bound to biotinylated testosterone coated on a streptavidin bead. This resulted in a number of NCS mutants with K_d values around 20 nM for the streptavidin-bound testosterone. This is a promising result as it shows that apoNCS is a protein scaffold that can be engineered to specifically bind molecules completely unrelated to the natural chromophore. Unfortunately, the K_d values for free testosterone were lower, between 7 and 55 μM. This illustrates one of the problems with this method at present, as the proteins are not just selected for their binding to testosterone, but also to the biotin spacer and the bound streptavidin.

Having engineered this new ligand selectivity into mutants of apoNCS, the authors went on to further investigate these systems by obtaining crystallographic data.[70] The poor solubility of testosterone in water led them to use a testosterone hemisuccinate derivative in its place. Surprisingly one of the mutants revealed that instead of binding a single testosterone species, the binding site had instead evolved to bind two such ligands (Figure 12). In contrast, in another mutant ligand binding induced dimerisation of proteins.[70] Thus, two testosterone hemisuccinates were bound between two proteins.

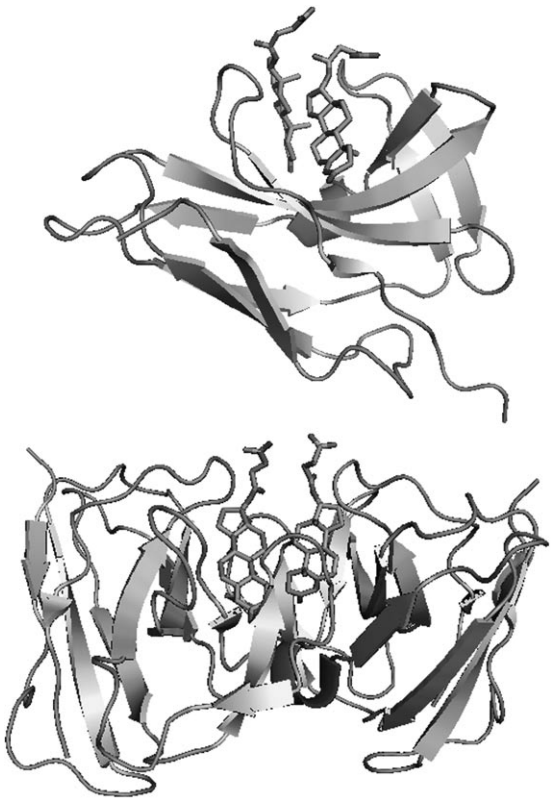


Figure 12. The crystal structures of two apoNCS mutants, selected by an in vitro evolution process to bind testosterone, bound to testosterone hemisuccinate. The first (PDB ID: 2CBO) has bound two such ligands, while the second (PDB ID: 2CBT) has formed a dimer, in which two ligands lie sandwiched between the two proteins.

The natural question that arises from these investigations is why the directed evolution process has led to the selection of proteins that bind more than one testosterone derivative. A very convincing hypothesis was put forward to explain this intriguing behaviour. Streptavidin is a tetramer, and because of its internal symmetry it would essentially display two biotinylated testosterone units on each face. Thus, the mutants could bind two testosterone molecules as this would lead to a much higher binding energy and would therefore be selected through an avidity effect. This also helps to explain the reduced binding strength of the free testosterone relative to the streptavidin-bound version, as in the free form two separate testosterone molecules are involved and thus a greater entropic penalty is involved. Nonetheless, this work highlights the exciting potential for the introduction of new binding specificities in the chromoproteins.

It is also worth noting that locations other than the binding pocket of NCS have been considered for modification in an attempt to introduce new binding selectivity. Nicaise et al. have noted that apoNCS has the same overall fold as immunoglobulins, and have grafted on to it the complementarity determining region 3 (CDR3) from the camel antilysozyme immunoglobulin.[71] The resulting apoNCS mutant successfully bound the

target lysozyme. This opens up the possibility of conferring specificity to apoNCS to target certain proteins, and thus when a drug is bound, to deliver it for attack.

7. Outlook

NCS is by far the most studied of the chromoproteins; however, each member of this intriguing class deserves investigation. Each of the apoproteins will have its own specificity to new ligands to probe, and each will have the varying methods by which they protect and deliver their corresponding chromophores to explore. A number of the chromoproteins are yet to have their holo complex structures determined, and indeed some of the chromophore structures remain elusive themselves. There is also still much uncertainty regarding whether the chromoproteins cross the cellular and nuclear membranes to deliver the reactive chromophores directly to the DNA, or whether the chromophore is released at the cell surface to make its own way across. A conclusive answer to this question will do much to clarify the mode of action of these complexes.

The continued efforts to test the scope of the apoproteins as generic drug-delivery systems will be particularly interesting in the future. The discovery of new ligands for the chromoproteins, such as the flavones, opens the possibility of using these molecules as anchors for the attachment of other drug motifs. The successful application of directed evolution to apoNCS has opened the door for new binding specificities to be introduced in the chromoproteins.

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Keywords: chromoprotein · drug delivery · enediyne · ligand · neocarzinostatin

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