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Design, synthesis and ribosome binding of chloramphenicol nucleotide and intercalator conjugates

Dorte Johansson,[a] Carsten H. Jessen,[a] Jacob Pøhlsgaard,[b] Kenneth B. Jensen,[a] Birte Vester,[b] Erik B. Pedersen[a] and Poul Nielsen[a,*]

[a]*Nucleic Acid Center*[†]*, Department of Chemistry, University of Southern Denmark, 5230 Odense M, Denmark*
[b]*Nucleic Acid Center*[†]*, Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark*

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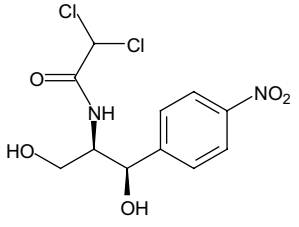
Abstract—Molecular modelling based on X-ray structures of the antibiotic drug chloramphenicol bound in a bacterial ribosome has been used for design of chloramphenicol derivatives. Conjugates of the chloramphenicol amine through appropriate linkers to either a pyrene moiety or to a mono- or dinucleotide moiety were designed to improve binding to ribosomes by providing specific interactions in the peptidyl transferase site or to the P-loop in the ribosome. Specific binding of the conjugates were investigated by footprinting analysis using chemical modifications of accessible nucleotides in ribosomal RNA. The pyrene chloramphenicol conjugate shows enhanced binding to the chloramphenicol binding site compared to the native chloramphenicol, whereas the four nucleotide conjugates could not be shown to bind to the chloramphenicol binding site or to the P-loop.

The presence of bacterial resistance to current antibiotics is a serious and widespread problem that motivates a search for new effective drugs. Most new antibiotics, however, are directed to well-established targets, for example, the bacterial ribosome. Studies of such ribosomal antibiotic sites are important tools for understanding the inhibitory mechanisms of drugs and for drug design. Recent reports of X-ray structures of many different antibiotics bound to the ribosomal subunits[1–3] support modelling and design of new drugs with presumed enhanced binding and increased specificity.

The antibiotic chloramphenicol (**1**) inhibits protein synthesis by binding to the peptidyl transferase region of the ribosome.[4] Its clinical use is limited because of toxic site effects. Chloramphenicol has been derivatised in many ways in order to define its essential functional groups and to improve its drug properties.[5,6] Elucidation of the X-ray structures of chloramphenicol bound to the 50S ribosomal subunit from two different organisms revealed two different binding sites of the drug.[1,2] Thus, there might be two chloramphenicol binding sites in the ribosome with the strongest binding site presumed to be the clinically relevant site,[7] and the site considered in this study.



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The high affinity chloramphenicol binding site is composed entirely of ribosomal RNA and overlaps the binding site of the antibiotic puromycin.[1,7] A transition state inhibitor consisting of a CCdA-p-puromycin conjugate based on a phosphoramidate linkage mimicking the peptidyltransferase reaction[8] was shown by X-ray crystallography to bind to G2252 and G2251 in the ribosomal P-loop by Watson–Crick base pairing to the two adjacent cytidines and to bind to the A-site with the pyromycin part.[9] This inhibitor displayed a strong activity as a peptidyltransferase inhibitor.[8] This result

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[†]Nucleic Acid Center is funded by the Danish National Research Foundation for studies on nucleic acid chemical biology.
* Corresponding author. Tel.: +45 6550 2565; fax: +45 6615 8780; e-mail: pon@chem.sdu.dk

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motivated us to explore the potential of similar but simpler and easily available conjugates of the chloramphenicol core structure to nucleotides with potential binding to the P-loop. Furthermore, in an alternative approach we envisioned that conjugation to a stacking moiety such as a pyrene might be another way of improving the target affinity of chloramphenicol. Related studies with conjugates, but without specific RNA targets have been described.[6,10] In both approaches, we applied the X-ray structural information of the ribosome in modelling experiment using the MacroModel system.[11]

From the known SAR of chloramphenicol[5] and the knowledge of the ribosomal binding site,[1] we decided to replace the dichloroacetyl group of chloramphenicol by alternative acyl groups as the anchor for conjugation. We designed and prepared four chloramphenicol nucleotide conjugates for improved and combined binding to the peptidyl transferase centre and the P-loop region of the ribosome as well as one chloramphenicol pyrene conjugate (Fig. 1).

Design of the pyrene conjugate **2** was done in MacroModel from the *D.radidurans*/chloramphenicol structure. However, no specific binding site is targeted, and no design requirements were placed on the linker between the components. A glycine linker, combined with the available (1-pyrenyl)acetic acid, was chosen as a synthetically convenient linker. On the other hand, the P-loop targeting conjugates needed a more precisely designed linker. The P-loop binding CC nucleotides from the *H.marismirtui*/CCdA-p-puromycin co-crystal[9] were moved into the *D.radidurans*/chloramphenicol structure[1] in MolMol. In dynamic simulations (performed in MacroModel using the amber* force field in implicit water) the two core components, that is, the chloramphenicol part and the dinucleotide, were fixed and joined by different short linkers. Four structures were subsequently chosen for synthesis providing two different synthetically convenient linkers and mono- as well as dinucleotide conjugates **3**–**6** (Fig. 1). The design of the two types of chloramphenicol conjugates and the envisioned ribosomal binding sites is visualised in Figure 2.

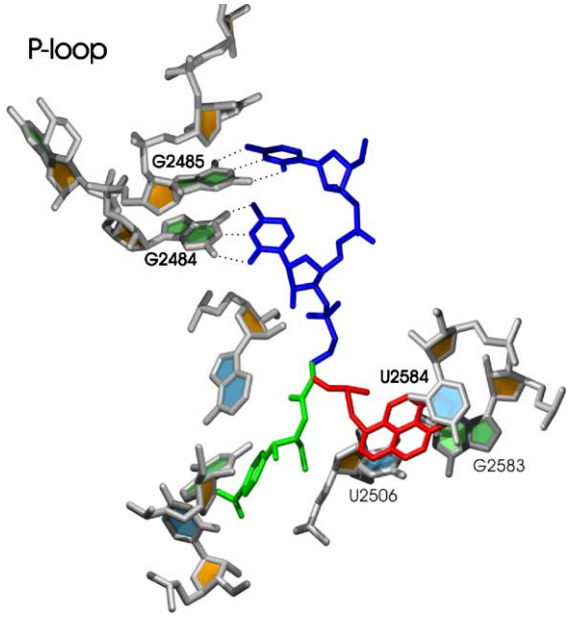


Figure 2. A combination of the two chloramphenicol conjugate types are shown with the chloramphenicol part in green, the pyrene (compound **2**) in red and two cytidine residues (compound **6**) in blue. Relevant 23S RNA nucleotides are shown as labelled (*E. coli* numbering). Predicted hydrogen bonds to P-loop nucleotides are indicated.

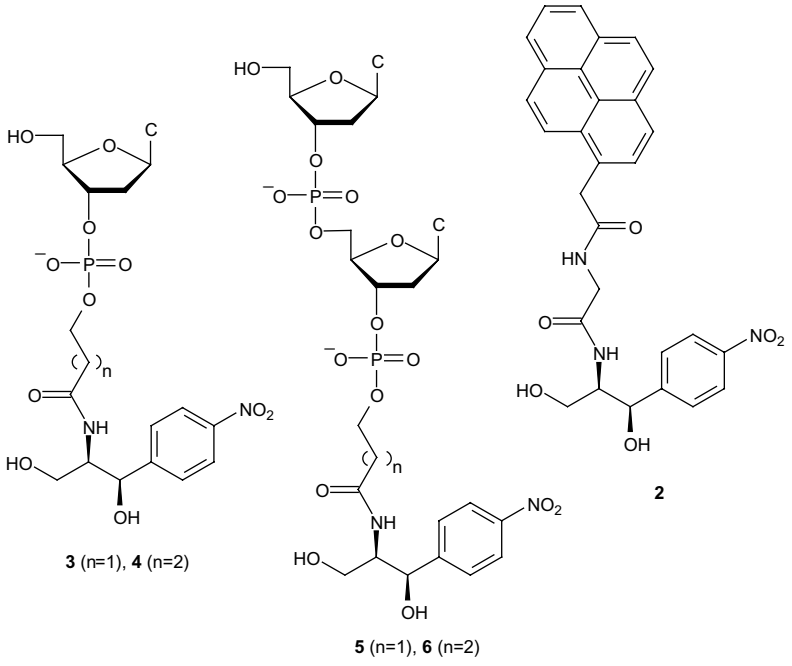
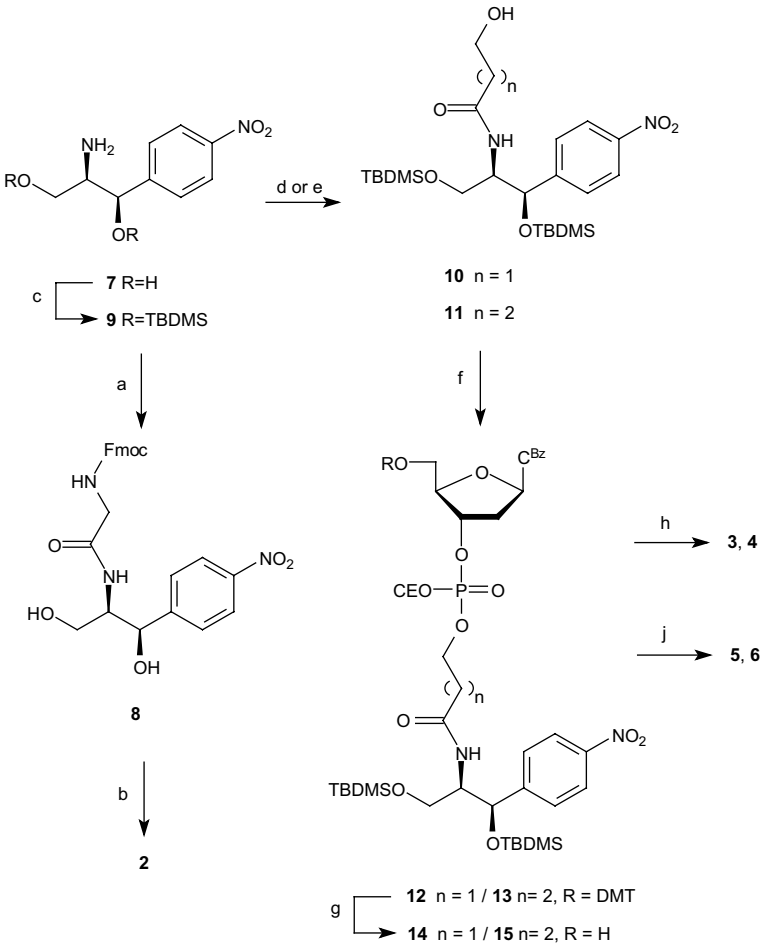


Figure 1. The designed pyrene and nucleotide chloramphenicol conjugates. C = cytosine-1-yl.

The commercially available precursor for chloramphenicol derivatives (1*R*,2*R*)-2-amino-1-(4-nitrophenyl)-1,3-propandiol (**7**) was used as a starting material for the preparation of the conjugates (Scheme 1). In order to prepare the pyrene conjugate with a glycine linkage, **2**, two subsequent coupling steps were performed. The appropriate Fmoc-protected glycine derivative was coupled with **7** to give **8** in a high yield. The Fmoc group was used after a similar Boc-strategy has failed, and the Fmoc-group was easily removed by a mild basic treatment. (1-Pyrenyl)acetic acid was converted to its *N*-hydroxyl succinimide ester in situ and mixed with the deprotected **8** to give the target conjugate **2** in a reasonable yield.

In order to form the nucleotide conjugates of the chloramphenicol amine, the alcohol functionalities of **7** were selectively protected as silyl ethers to give **9** in a high yield (Scheme 1). For the preparation of the appropriate linkers, **9** was reacted with either β-propiolactone or γ-butyrolactone to give **10** and **11**,[12] respectively. Thus, γ-butyrolactone was simply used as the solvent affording **11** in a high yield, whereas β-propiolactone surprisingly was less reactive and in a basic solution afforded **10** in a lower but acceptable yield. The primary alcohols of **10** and **11** were used for the standard nucleotide coupling reactions[13] with a commercial cytidine phosphoramidite that contains a DMT-protection for the 5′-hydroxyl group and a benzoyl group to protect the exocyclic amine of the nucleobase. Thus, the multiple protected 3′-nucleotide chloramphenicol conjugates **12** and **13** were obtained. Subsequent detritylation afforded compound **14** and **15**, respectively. A complete deprotection using TBAF for the removal of the silyl-groups and aqueous ammonia for the removal of the cyanoethyl group and the *N*-benzoyl group afforded after an HPLC-purification the target conjugates **3** and **4**. On the other hand, a second coupling reaction using **14** and **15** and the cytosine phosphoramidite followed by the standard deprotection steps and HPLC-purification afforded the target dinucleotide conjugates **5** and **6**. The constitution of the conjugates **3**–**6** was verified by MS and by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy.[14]

The interaction of the chloramphenicol derivatives with the peptidyl transferase centre of *Escherichia coli*



Scheme 1. Reagents and conditions: (a) Fmoc-Gly-OC_6F_5, CH_3CN, 95%; (b) i, Et_3N, DMF; ii, (1-pyrenyl)acetic acid, DCC, NHS, DMF, 39%; (c) TBDMS-OTf, 2,6-lutidine, CH_2Cl_2, 94%; (d) β-propiolactone, 2,6-lutidine, CH_2Cl_2, **10** 24%; (e) γ-butyrolactone, **11** 66%; (f) dC-CE/DMT phosphoramidite, 1H-tetrazole, CH_3CN *then* I_2, aq pyridine, **12** 48%, **13** 34%; (g) TFA, $CHCl_3$, Et_3SiH, CH_2Cl_2, **14** 45%, **15** 54%; (h) i, TBAF, THF; ii, 32% aq NH_3, HPLC, **3** 38%, **4** 58%; (j) i, dC-CE/DMT phosphoramidite, 1*H*-tetrazole, CH_3CN *then* I_2, aq pyridine, 30%, 28%; ii, TFA, $CHCl_3$, Et_3SiH, CH_2Cl_2, 52%, 52%; iii, TBAF, THF; iv, 32% aq NH_3, HPLC, **5** 47%, **6** 70%. C^{Bz} = *N*-4-benzoylcytosine-1-yl, TBDMS = *tert*-butydimethylsilyl, CE = 2-cyanoethyl, DMT = 4,4′-dimethoxytrityl.

ribosomes were investigated by footprinting. The drug binding sites were studied using chemical probing, by which accessible nucleotides are modified.[15] After binding of chloramphenicol and the chloramphenicol conjugates to 70S ribosomes, the antibiotic–ribosome complexes and free ribosomes were treated with CMCT [1-cyclohexyl-3-(2-*N*-(*N*-methyl)morpholinoethyl)carbodiimide] to probe the N3 of uridine and N1 of guanosine residues. Some of the complexes were also treated with kethoxal to probe the N1 position of guanosine. Primer extension with reverse transcriptase was then used to identify the alterations in chemical reactivity of specific nucleotides induced by the binding of the drug to the ribosome. Growth of *E. coli* cells, isolation of ribosomes as well as modifications and primer extensions were performed essentially as described before.[16,17] Figure 3 shows an autoradiogram of primer extension of the region where CMCT modification of nucleotide U2506 is known to be protected by chloramphenicol.[18] The protection effects seen from **1** (chloramphenicol) and **2** were quantified by phosphor image scanning. The effect shows that **2** binds to the peptidyl transferase part of the ribosome and probably with the choramphenicol part of the molecule occupying the same site as chloramphenicol. Furthermore, it shows a binding relative to chloramphenicol clearly enhanced by at least an order of magnitude. Inspection of the X-ray structure of the ribosomal drug binding site suggests that the pyrene part of **2** could intercalate or interact by stacking with nucleotide U2506, G2583 and U2584 (Fig. 2). As U2584-5 are also accessible for CMCT modification this region was also explored by footprinting but no protection effect were observed here. Thus, any interaction between the pyrene of **2** and nucleobases has not been clearly demonstrated.

A similar analysis of binding of the four nucleotide conjugates **3**–**6** to ribosomes showed no protection effect at U2506. Apparently, the chloramphenicol part of these molecules does not bind similarly to chloramphenicol. However, it was still a possibility that the compounds interact with the P-loop in ribosomal 23S RNA on the ribosome. Thus, additional experiments with kethoxal modifications were performed searching for base pairing from compound **3**–**6** to the P-loop but, no effects on the modification pattern were seen in this region. To explore the possibility of other footprints, the A-loop in ribosomal 23S RNA were analysed by primer extension after CMCT modification of ribosome complexes. Re-examining the model of the compounds docked into the 50S ribosomal X-ray structure to propose explanations for the missing footprints suggests that the linker regions between the choramphenicol part and the nucleotide part may be too short or too rigid to allow combined binding at two sites. As the binding of choramphenicol by itself is not very strong, the burden of a 'no affinity affording nucleotide part' may be too heavy.

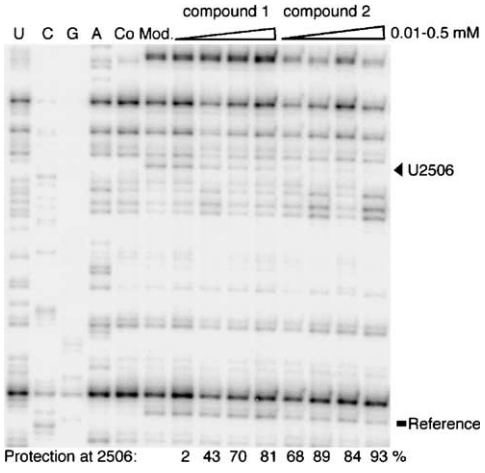


Figure 3. Gel analysis of protection of CMCT modification at nucleotide 2506. Lane U, C, G, A indicate the 23S RNA sequence in the analysed region. Co denotes a ribosomal RNA control lane. Mod. denotes RNA from CMCT modified ribosomes. The triangles are from ribosomes complexed with compounds at 0.01, 0.05, 0.2 and 0.5 mM, respectively, and treated with CMCT. The protections are normalised to the indicated reference band.

In conclusion, the nucleotide conjugate targets **3**–**6** were successfully synthesised by the application of solution phase phosphoramidite chemistry. Footprinting were performed to determine the binding of the conjugates in the peptidyl transferase centre of the ribosome as anticipated by modelling experiments. The chloramphenicol pyrene conjugate **2** shows protection of U2506 indicating binding to the chloramphenicol site and the concentration dependent protection shows an significantly enhanced binding relative to chloramphenicol. In contrary, no binding was found for the chloramphenicol nucleotide conjugates **3**–**6**. This might be due to linker restrictions, however, and further adjustment of the linker structure might be sufficient for obtaining more efficient compounds. Thus, the binding of the pyrene derivative demonstrates the possibilities for large choramphenicol conjugates to bind to the drug site. Further effort in the design and synthesis of chloramphenicol nucleic acid conjugates is in progress. Clearly, the potential of using intercalating moieties in conjugates with antibiotics is a promising approach.

Acknowledgements

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