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# Anthraquinone–Oligodeoxynucleotide Conjugates as Inhibitors of Gene Transcription

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**Abstract**

Several anthraquinone–oligodeoxynucleotide conjugates have been synthesized and their inhibition of transcription of the *bla* gene in *E. coli* has been determined.

All conjugates at 10 $\mu$M inhibited transcription and three conjugates inhibited mRNA production by 40 to 50% for periods of up to 60 min.

It has been known for several years that oligodeoxynucleotides form sequence-specific Hoogsteen hydrogen-bonds with double-helix DNA, resulting in the formation of a triple helix (Hoogsteen 1959). Interest in these compounds has increased since it was shown that sequence-specific triple-helix formation can inhibit the transcription of selected genes (Hélène & Toulme 1990). Most work has concentrated on targeting homopyrimidine oligodeoxynucleotides at a homopurine DNA sequence because of the greater stability of the resulting triple helix (Hélène 1991). The use of other bases in the oligodeoxynucleotides can lead to distortion of the DNA backbone and a concomitant decrease in the stability of the triple helix, whereas cytosine requires protonation to enable it to form Hoogsteen hydrogen-bonds. This means that the triple-helix-forming properties of oligodeoxynucleotides containing cytosine are pH-dependent. The presence of adjacent protonated cytosines in the oligo-deoxynucleotide sequence leads to distortion of the triple-helix backbone and instability as a result of electrostatic repulsion.

The attachment of a polycyclic intercalator to either the 3′ or 5′ end of the oligodeoxynucleotides increases the stability of any triplex formed, although the effect is more pronounced at the 5′ end (Sun et al 1989). This effect is attributed to the presence of the hydrophobic chromophore intercalating between the base pairs of DNA to anchor the oligodeoxynucleotides in place and enable the formation of a stable triple helix.

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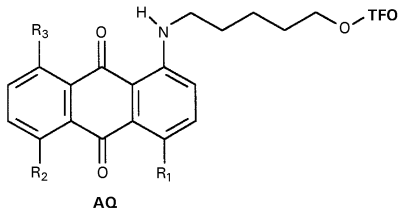
Intercalator–oligodeoxynucleotide complexes of this type enable the use of oligodeoxynucleotides to target a cytotoxic compound to a particular DNA sequence with the aim of inhibiting the transcription of selected genes. Agents of this type should have significant advantages over current anticancer therapy which targets DNA non-specifically.

As part of our drug-design programme of investigation of agents which target DNA sequence-specifically, several anthraquinone derivatives (Table 1) based on mitoxantrone, a clinically effective anticancer agent with the capacity to intercalate with DNA, were synthesized in high yield from chloroanthraquinones and fully characterized (Gibson et al 1996a). The 1-, 1,4-, 1,5- and 1,8-substituted anthraquinones were attached to a 13mer oligodeoxynucleotide, 5′-d(CTTTTTCCTTCTC)-3′, via a phosphoramidite intermediate and the capacity of the anthraquinone–oligodeoxynucleotide complexes to inhibit gene transcription was determined. The sequence chosen is complementary to a homopurine sequence located just downstream of the promoter site of the $\beta$-lactamase gene in *E. coli*, a gene target for which a simple assay already exists (Duval-Valentin et al 1992).

The effect on triple-helix stability of replacing the cytosines in the oligodeoxynucleotides with 5-methylcytosine was also investigated. Preliminary studies using computer modelling and DNAse footprinting techniques were undertaken to determine the sequence specificity of triplex formation and the optimum structure of the anthraquinone–oligodeoxynucleotides linker (Gibson et al 1996b, 1997).

Table 1. The anthraquinone (AQ)-oligodeoxynucleotide complexes.




AQ

| | |
|---|---|
| TFO11, TFO13 | $R_1 = R_2 = R_3 = H$ |
| TFO14 | $R_1 = R_3 = H$; $R_2 = NHCH_2CH_2N(CH_2CH_3)_2$ |
| TFO15 | $R_1 = R_2 = H$; $R_3 = NHCH_2CH_2N(CH_2CH_3)_2$ |
| TFO10 | 5′-CTTTTTCCTTCTC-3′ |
| TFO11 | 5′-AQ-CTTTTTCCTTCTC-3′ |
| TFO12 | 5′-$^{Me}$CTTTTT$^{Me}$C$^{Me}$CTT$^{Me}$CTC-3′ |
| TFO13 | 5′-AQ-$^{Me}$CTTTTT$^{Me}$C$^{Me}$CTT$^{Me}$CTC-3′ |
| TFO14 | 5′-AQ-$^{Me}$CTTTTT$^{Me}$C$^{Me}$CTT$^{Me}$CTC-3′ |
| TFO15 | 5′-AQ-$^{Me}$CTTTTT$^{Me}$C$^{Me}$CTT$^{Me}$CTC-3′ |

For the gene-transcription assays, a series of dose-dependent experiments was conducted at anthraquinone–oligodeoxynucleotide concentrations from 0·1 to 100 $\mu$M to determine the concentration range for inhibition of transcription. The most effective inhibitors were then evaluated between 30 s and 10 min, and then between 10 min and 60 min, to determine the duration of the inhibition.

## Materials and Methods

*Synthesis of anthraquinone–oligodeoxynucleotide conjugates*

Substituted anthraquinones and their phosphytilated analogues were synthesized according to the procedure of Gibson et al (1996a). The synthesis of the oligodeoxynucleotides and attachment of the oligodeoxynucleotides to phosphytilated anthraquinones were undertaken by Oswel DNA, University of Southampton, Southampton, UK.

*Preparation of target DNA fragment*

This was achieved by the method of Hartley & Wyatt (1997). Briefly, a 356-base-pair fragment carrying the *bla* promoter was isolated from plasmid pBR322. The polymerase chain reaction (PCR) product of this fragment was generated to produce a large quantity of the target DNA fragment.

*In-vitro transcription assay*

Transcription assays were performed in pH 7 buffer containing Tris HCl (40 mM), $MgCl_2$ (6 mM), spermidine (2 mM) and NaCl (10 mM). The prepared DNA (5 nM) was added, then DTT (dithiothreitol; 10 mM), bovine serum albumin (BSA; 100 $\mu g\,\mu L^{-1}$), RNA polymerase (30 nM) and Rnasin (1 U $\mu L^{-1}$) and the mixture was incubated in transcription buffer for 45 min at 37°C. Heparin was added to a final concentration of 150 $\mu g\,mL^{-1}$ and 1 min later the oligodeoxynucleotide (100 $\mu$M) was added and the mixture was incubated at 25°C for 10 min.

Final nucleotide concentrations were 500 $\mu$M for ATP, GTP and CTP and 10 $\mu$M for UTP, including [$\alpha$-$^{32}$P] UTP at 20 Ci $mmol^{-1}$. Each solution was incubated at 25°C, followed by termination in dry ice and two ethanol precipitations. The resulting samples were resuspended in formamide gel loading buffer, heated to 95°C for 2 min, then loaded on to a 6% denaturing polyacrylamide gel.

## Results and Discussion

Data obtained from 10 $\mu$M concentrations of the anthraquinone–oligodeoxynucleotide complexes are shown in Figure 1. The figure shows the inverse band intensity of mRNA production, hence the longer the bar, the lower the intensity of the transcription band. TFO13, TFO14 and TFO15 significantly inhibited transcription compared with the control. These trends were present at the higher concentration of 100 $\mu$M, although at 0·1 $\mu$M detectable inhibition of transcription was observed for TFO13, TFO14 and TFO15 only.

All the anthraquinone–oligodeoxynucleotide conjugates inhibited transcription to some extent. Inhibition by TFO13, 14 and 15 was significant compared with scrambled oligodeoxynucleotides used as controls (Figure 1). Very little inhibition of mRNA production was observed for TFO12, despite the complementarity of its sequence with that of the target DNA. The scrambled oligodeoxynucleotides inhibited mRNA similarly, confirming previous foot-printing studies which showed free oligodeoxynucleotides form transient and weak triple helices (Gibson et al 1996a). The role of the anthraquinone seems to be that of a molecular anchor which intercalates at the triplex–duplex junction and stabilizes the triple helix (Gibson et al 1997). These data also show TFO15 to be a weaker inhibitor of mRNA formation than TFO13 and TFO14. This might be because of weaker interaction of this 1,8-disubstituted anthraquinone derivative, compared with the 1- and 1,5-substituted compounds. Almost no inhibition of transcription was observed for TFO11, a mono-substituted anthraquinone with non-methylated cytosines in the oligodeoxynucleotide. This was attributed to the reduced stability of triplex-containing free cytosine, as discussed below. The

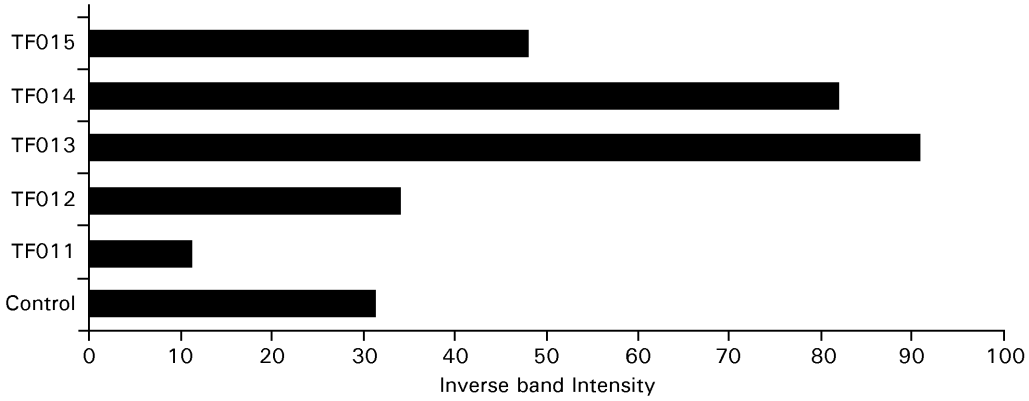



Figure 1. Inhibition of gene transcription by anthraquinone–oligodeoxynucleotide complexes (10 $\mu$M). Densitometry data from full-length RNA transcripts.

strongest inhibition of mRNA production was shown by TFO13 and TFO14. This led us to conclude that once linked to an oligodeoxynucleotide a second cationic side-chain on the anthraquinone had little effect on the stabilization of the triple helix and hence inhibition of mRNA production. This result was unexpected because we expected a cationic side-chain to enhance the stability of the triplex as a result of electrostatic interactions with the anionic phosphate backbone of the DNA.

*Methylcytosine replacement*

The binding of TFO11 was compared with that of TFO13 in an attempt to determine the effect, if any, of replacing the cytosines of the oligodeoxynucleotides with 5-methylcytosine. Previous studies have shown that substitution of cytosine with 5-methylcytosine can increase the pH range of triplex formation almost to neutrality (Lee et al 1984; Collier et al 1991). The effect is attributed to a slight increase in $pK_a$ and the hydrophobic action of the helical spine of methyl groups present in the major groove.

The data in Figure 1 support this view and show TFO13 to be a much better inhibitor of transcription than the corresponding demethyl compound, TFO11.

Compounds TFO13 and TFO14, the most effective inhibitors, were investigated over a time course of 0·5 to 10 min, with target DNA as a control. The results obtained are shown in Figure 2. The presence of TFO14 reduced the amount of mRNA formed by 50% (approx.) and in the presence of TFO13 only 25% (approx.) mRNA was produced after 10 min.

TFO13 and TFO14 were then compared with TFO12 and a DNA control over a time period of 10 to 60 min, thus enabling direct comparison of mRNA production for unmodified oligodeoxynucleotide (TFO12) with that of anthraquinone-derivatized oligodeoxynucleotides (TFO13 and TFO14). The results are presented in Figure 3 and show two main features—TFO12 leads to reduced transcription, compared with the control, for up to

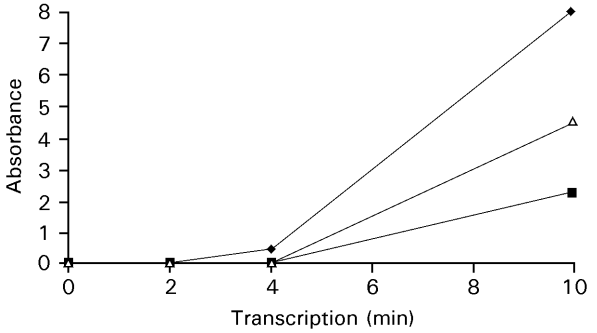



Figure 2. Inhibition of gene transcription by anthraquinone-oligodeoxynucleotide complexes between 0·5 and 10 min: ◆, control; ■, TFO13; Δ, TFO14.

20 min, beyond which time the transcription pattern of the TFO12 sample was similar to that of the control. This suggests that free oligodeoxynucleotides, even with all the cytosine bases replaced by methylcytosine, become ineffective at inhibiting transcription after a relatively short period, and that any inhibition seen with free oligodeoxynucleotides is reversible, with levels of mRNA returning to control values after 20 min. More interestingly, TFO13 and TFO14 significantly reduced mRNA production for the 60-min duration of the experiment. Again, the 1-substituted anthraquinone–

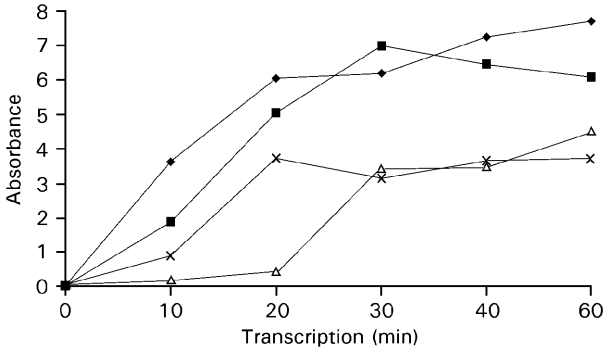



Figure 3. Inhibition of gene transcription by anthraquinone–oligodeoxynucleotide complexes over a 60-min period: ◆, control; ■, TFO12; Δ, TFO13; ×, TFO14.

oligodeoxynucleotide (TFO13) seemed to be the most effective inhibitor, resulting in almost total inhibition of gene transcription for the first 20 min of the assay. We are forced to conclude that the presence of a second cationic side-chain is not beneficial, and might even be detrimental to triplex formation. Our modelling studies (Gibson et al 1996b, 1997) revealed that a perpendicular mode of intercalation was energetically preferred (i.e. the long axis of the anthra-quinone intercalates perpendicular to the base pair axis). This is supported by the results from the 1,8-disubstituted anthraquinone–oligodeoxynucleotide (TFO15) which, because of its substitution pattern, requires a parallel mode of intercalation (Islam et al 1985) and inhibits transcription less than do the other substituted anthraquinone–oligodeoxy-nucleotides. The 1,5-disubstituted anthraquinone-oligodeoxynucleotide (TFO14) intercalates in a perpendicular orientation, which requires local melting of the DNA to enable the side-chains to reside in both the major and minor grooves (Islam et al 1985). Such local melting will be less favourable in triplex DNA than in duplex DNA and might explain the reduced capacity of TFO14 to inhibit transcription. TFO13 can intercalate in the preferred perpendicular orientation without the requirement for local melting of the DNA, giving it the capacity to form a stable triple helix and resulting in the largest inhibition of gene transcription for the longer duration.

We therefore conclude that we have successfully synthesized anthraquinone–oligodeoxynucleotide conjugates which, in our assay, can inhibit transcription of the $\beta$-lactamase gene in *E. coli* for periods up to 60 min. These results are in agreement with our previous molecular modelling studies on anthraquinone–oligodeoxynucleotide complexes as triple helix-stabilizing agents.

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