Improving Antisense Oligonucleotide Binding to Human Serum Albumin: Dramatic Effect of Ibuprofen Conjugation

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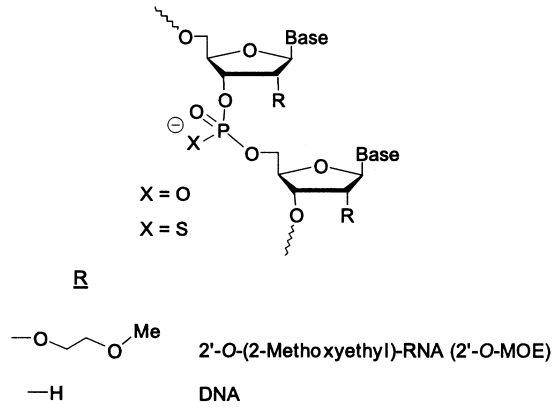
antisense oligonucleotides · nucleic acids · oligonucleotides · protein binding · therapeutics

The interaction of antisense oligonucleotides with serum and cellular proteins determines their pharmacokinetic (transport to and distribution in target tissues) and pharmacodynamic (binding to the mRNA target) properties and hence their eventual pharmacology.[1] In general, binding of drugs to serum albumin, α_2-macroglobulin, immunoglobulins, and lipoproteins in the bloodstream governs their transport and tissue distribution.[2] The first generation antisense compounds, 2′-deoxyphosphorothioate oligonucleotides (containing P=S linkages, where R = H and X = S in Scheme 1), bind rapidly to serum and cellular



Scheme 1. *General structure of the antisense oligonucleotides used in this study.*

proteins and thus have favorable pharmacokinetic properties.[1, 3–6] However, these P=S oligonucleotides also bind to proteins such as thrombin, Factor IX, and Factor H. This binding probably contributes to the undesirable dose-limiting side effects of these compounds in the clinical setting, side effects such as prolonged clotting time and complement activation.[7, 8] To make safer and more effective oligonucleotide drugs, it would be valuable to enhance the interaction of these molecules with proteins involved in transport and absorption and to minimize the interaction with proteins responsible for their side effects.

Changing the P=S linkages to the native phosphodiester (P=O) linkages overcomes the above side effects and increases the binding affinity to the target RNA;[9, 10] however, this change also results in the loss of nuclease resistance and, consequently, in a more rapid degradation of the drug.[11] The 2′-*O*-(2-methoxyethyl) modification (2′-*O*-MOE, R = 2′-*O*-MOE, X = O in Scheme 1) has been shown to improve binding affinity to the target mRNA and to limit the undesired sensitivity to nucleolytic degradation.[12–14] Unfortunately, the replacement of P=S linkages by P=O linkages results in poor pharmacokinetic properties, such as limited distribution to organs and faster urinary elimination, presumably due to the lack of binding to serum proteins.[15] It would therefore be highly desirable to improve binding affinity of non-phosphorothioate compounds for human serum albumin.

Human serum albumin (HSA), a water-soluble protein of 585 amino acids with a molecular weight of 66 kD, is the most abundant protein in plasma (3.5 – 5.0 g per 100 mL in blood plasma), but it also exists in lower concentrations in extra vascular fluids. It has a large number of charged amino acids (about 100 negative charges and 100 positive charges), with an isoelectric point of 5.0 and a net negative charge of -15 at a plasma pH value of 7.4, and it attracts both anions and cations.[16–18] The antiinflammatory small-molecule drug ibuprofen binds to the domain II A of HSA with a binding affinity of 10^{-6} M.[19] We have attempted to improve the binding affinity of antisense oligonucleotides to serum albumin by chemically conjugating ibuprofen to the oligonucleotides.

As shown in Scheme 2, (*S*)-(+)-ibuprofen (Sigma) was attached to 3′-*O*-(6-aminohexyl)-5′-*O*-dimethoxytrityluridine[20, 21] by using the pentafluorophenyl ester derivative of ibuprofen. The resulting conjugate was hemisuccinylated at the 2′-position and attached to long chain aminoalkyl controlled pore glass (LCAA-CPG). Oligonucleotide synthesis was carried out by starting with this solid support and using either the 2′-*O*-MOE or the 2′-deoxynucleoside nucleoside β-cyanoethylphosphoramidites and standard automated DNA synthesis protocols.

To measure binding affinity, the 5′-end of each oligonucleotide was labeled with ^{32}P by using T4 polynucleotide kinase in standard procedures. The unincorporated label was removed through a G25 column and labeling was confirmed by polyacrylamide gel electrophoresis. A fixed concentration of labeled oligonucleotide (50 nM) was incubated with increasing concentrations of albumin (human fatty acid free serum albumin, Sigma A3782, lot 94H9318, Sigma Chemicals, St. Louis, MO) and incubated at 25 °C for one hour in phosphate-buffered saline buffer containing 0.1 mM ethylenediaminetetraacetate (EDTA) and 0.005% Tween 80. After incubation, the samples were loaded onto low-binding, regenerated cellulose filter membranes with a molecular weight cut-off of 30 000 (Millipore). The samples were spun gently in a microfuge (NYCentrifuge 5415C; Eppendorf, Westbury, NY) at 3000 rpm (735 g) for 3 – 6 minutes, thereby collecting ≈20% of the loaded volume in the filtrate.

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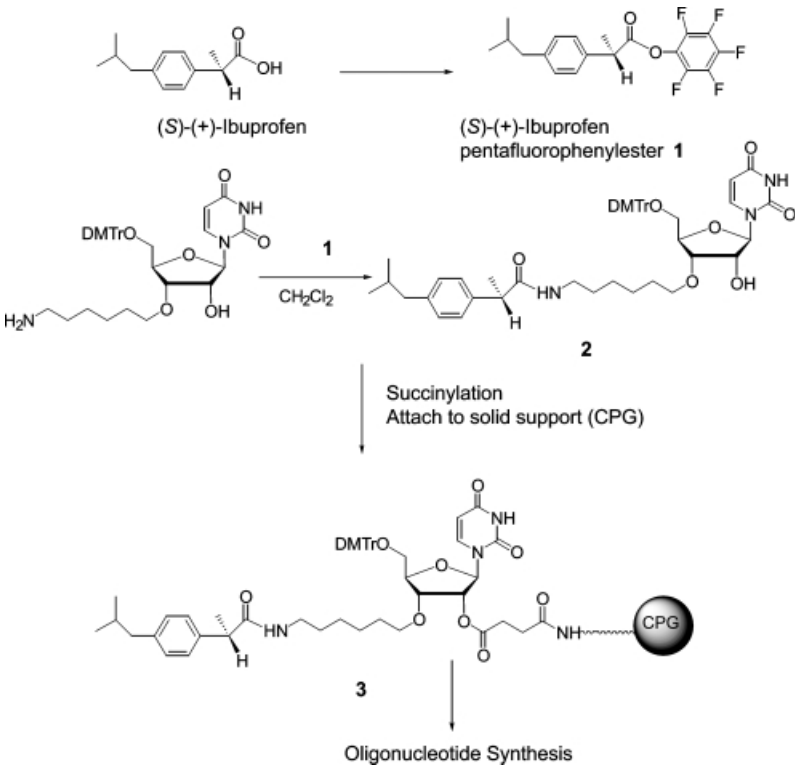

Scheme 2. *Conjugation of the pentafuorophenyl ester derivative of ibuprofen to 3′-O-(6-aminohexyl)-5′-O-dimethoxytrityluridine and attachment of the resulting conjugate to the solid support. DMTr = dimethoxytriphenylmethyl (or dimethoxytrityl).*

Radioactivity present in aliquots from the filtrate and the initial (unfiltered) solutions was measured with a scintillation counter (model LS6000IC, Beckman, Fullerton, CA). The counts obtained in the filtrate aliquots represent the free (unbound) oligonucleotide, and appropriate calculations were performed to obtain the concentration of free oligonucleotides. Further calculations yielded the concentration of oligonucleotide bound to protein.[22, 23]

The extent of oligonucleotide binding to albumin was determined by use of an equilibrium filtration method. The fraction of bound oligonucleotide was plotted against the total albumin concentration. The equilibrium dissociation constant K_d was determined from nonlinear regression analysis of the fraction of oligonucleotide (ODN) bound (f_{bound}) as a function of the free albumin monomer concentration (f_{free}). The concentration of albumin monomer in solution was calculated by using $K_d = 150\ \mu M$ for the monomer–dimer equilibrium.[16, 17] A low concentration of the oligonucleotide relative to albumin allowed for detection of binding to only the tightest binding site on the albumin. Thus, the data (Figure 1) could be fittted to the two-state model described in Equation (1) where O is the unbound oligonucleotide, A is the unbound albumin, (OA) is the oligonucleotide–albumin complex, and K_A is the equilibrium association constant.

$$O + A \overset{K_A}{\leftrightarrow} (OA) \qquad (1)$$

The oligonucleotides tested are listed in Table 1. As seen in Figure 1 and Table 1, the phosphodiester oligonucleotides (both 2′-deoxy, I-8651, and 2′-*O*-MOE, I-11158) bound to HSA with extremely weak affinity ($K_D > 400\ \mu M$) while the phosphorothioate oligonucleotides, I-3067 and I-3082, had much greater affinity ($K_D = 7$ and $4\ \mu M$, respectively). Conjugation of an ibuprofen to the 3′-end of a phosphodiester oligonucleotide, either the unmodified 2′-deoxy (I-22955) or the 2′-*O*-MOE (I-27700) derivatives, increased the affinity to the range typical for phosphorothioate oligonucleotides ($K_D = 8\ \mu M$).

Ibuprofen also has an effect on the binding capacity of oligonucleotides to albumin. Capacity curves (Figure 2) were measured by using a technique similar to that used for the binding curves except that a fixed concentration of albumin ($50\ \mu M$) was employed and the concentration of labeled oligonucleotide was varied. As can be seen in Figure 2, a binding ratio of 0.75:1 (oligonucleotide:albumin) was measured for the conjugate, I-27700. A ratio of 0.2:1 was observed for the unconjugated 2′-deoxy phosphorothioate compound, I-3067. Thus, the capacity of HSA for the ibuprofen conjugate was dramatically greater than that of the unconjugated phosphorothioate oligonucleotide, a drug that binds well.

The enhanced binding shown by the ibuprofen-conjugated oligonucleotide for HSA was *not*

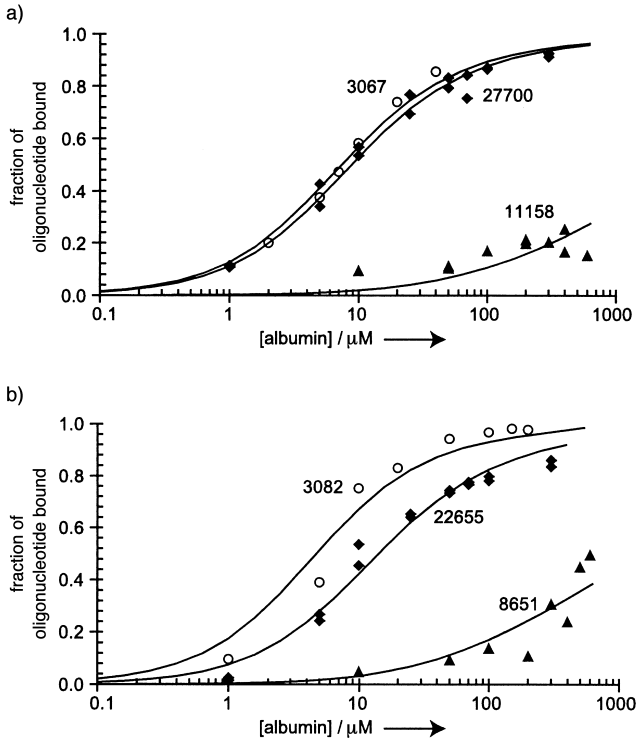

Figure 1. *Comparison of HSA binding of two ibuprofen conjugates (◆) to unconjugated controls (▲). Binding curves for the phosphorothioate DNA analogues of each sequence are also shown (○). The oligonucleotide (50 nM) was incubated with increasing concentrations of HSA as described in the text. The sequences of the compounds tested (labeled here with compound numbers) can be found in Table 1.*

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Table 1. *Oligonucleotides used in the study and equilibrium dissociation constants for binding to HSA.*

Isis number	Sequence	Chemistry	Conjugated ligand	K_D [μM][a]
I-27700	TCTGAGTAGCAGAGGAGCCT	2′-*O*-MOE/P=O	3′-linker–ibuprofen	8
I-11158	TCTGAGTAGCAGAGGAGCCT	2′-*O*-MOE/P=O	none	>400
I-3067	TCTGAGTAGCAGAGGAGCCT	2′-deoxy/P=S	none	7
I-22655	TGCATCCCCCAGGCCACCAT	2′-deoxy/P=O	3′-linker–ibuprofen	12
I-8651	TGCATCCCCCAGGCCACCAT	2′-deoxy/P=O	none	>400
I-3082	TGCATCCCCCAGGCCACCAT	2′-deoxy/P=S	none	4

[a] Equilibrium dissociation constants were obtained from the data in Figure 1 as described in the text.

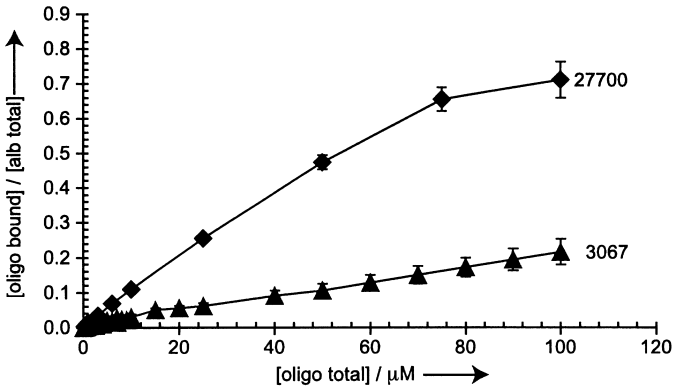

Figure 2. *Capacity of HSA for an ibuprofen conjugate I-27700 (◆) compared to that of an unconjugated phosphorothioate DNA, I-3067 (▲). Capacity was measured at 50 μM HSA with increasing concentrations of the oligonucleotide. The sequences of the compounds tested (labeled here with compound numbers) can be found in Table 1.oligo = oligonucleotide, alb = albumin.*

observed when the experiment was repeated with the plasma protein thrombin. Thrombin is a plasma protein known to bind phosphorothioate oligodeoxynucleotides with low nanomolar affinity.[24] The interaction between thrombin and oligonucleotides has been postulated to be responsible for prolongation of coagulation observed after treatment with phosphorothioate oligodeoxynucleotides.[25] Radiolabeled I-11158 or I-22270 (20 pM) were incubated with increasing concentrations of thrombin, and the binding was evaluated. Neither molecule bound to thrombin until the concentration of protein approached low millimolar concentrations (Figure 3).

Ibuprofen was conjugated to the oligonucleotide through the carboxyl group on the small molecule. If this group were critical for the interaction between ibuprofen and albumin, the conjugate would be expected to bind less tightly to albumin than free ibuprofen does. As the K_d values of the ibuprofen – oligonucleotide conjugate and ibuprofen are the same, there is no evidence that conjugation changes the mode of binding. It appears to be mainly the hydrophobic interaction between the aromatic ring of the ibuprofen and the aliphatic substituents on albumin that are important for this molecular recognition.[26]

The observed improvements in affinity and capacity of oligonucleotide binding to albumin were independent of the oligonucleotide sequence and are probably due to specific molecular recognition events between ibuprofen and albumin, rather than to the resultant small change in hydrophobicity of the oligonucleotide. In fact, conjugation of more lipophilic molecules such as cholesterol or pyrene did not result in such marked improvement in albumin binding while other arylpropionic acid ibuprofen analogues showed similar binding (data not shown).

This is the first example of a small-molecule conjugate that modulates the binding of antisense oligonucleotides to serum albumin. Since serum albumin is the major transport protein, ibuprofen conjugation is expected to alter dramatically the tissue distribution of oligonucleotides. In vivo pharmacokinetic analysis and tissue distribution experiments are in progress to confirm this hypothesis. However, at this time, further details regarding the binding site of the ibuprofen – oligonucleotide conjugate, the mechanism of interaction, the specificity of this interaction, and the toxicity of the conjugate are not known. Since the observed binding affinity is in the micromolar range, the release of the oligonucleotide from the protein is not an issue. Since it is a 3′-terminal conjugate the Tm to the target RNA should not be affected while the exonuclease resistance will be increased.[35] It is reasonable to expect that conjugation of

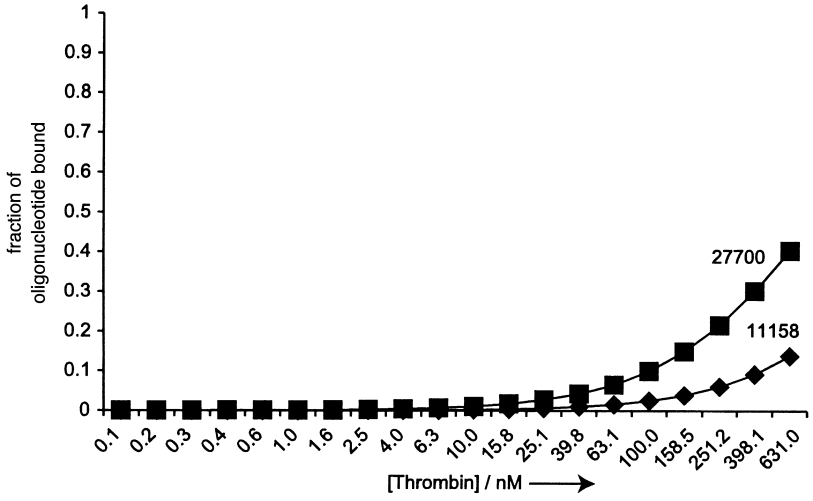

Figure 3. *Binding curves for thrombin binding to unconjugated 2′-O-MOE derivative I-11158 and its ibuprofen conjugate I-27700. The oligonucleotide (20 pM) was incubated with increasing concentrations of thrombin as described in the text. The sequences of the compounds tested (labeled here with compound numbers) can be found in Table 1.*

ibuprofen to other non-phosphorothioate oligomeric constructs, including peptide nucleic acids,[27–29] methyl phosphonates,[30] morpholinos,[31] the methylene(methylimino) backbone modification,[32] and phosphoramidates[33] and locked nucleic acids,[34] may also enhance the pharmacokinetic and tissue distribution properties of these constructs.

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A Signal Released by an Endophytic Attacker Acts as a Substrate for a Rapid Defensive Reaction of the Red Alga *Chondrus crispus*

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amino acids · biosynthesis · chemical defense · hydrogen peroxide · plant–pathogen interaction

Signal reception and regulation of physiological responses by macroalgae are only poorly understood to date. Besides some remarkable exceptions from the pheromone field[1] and the defensive reactions of certain brown algae,[2] only phenomenological descriptions of reactions to environmental cues have been given. The existing evidence nevertheless indicates that brown[3] as well as red[4] algae have developed elaborate strategies to detect the presence of herbivores or pathogens in their aqueous environment. Observed reactions include the immediate release of hydrogen peroxide or halogenated low-molecular-weight hydrocarbons as well as a long-term up-regulation of defensive metabolites. Comparable reactions are well known in higher plants. First evidence indicates that the underlying principles of signal recognition in algae differ significantly from those observed in higher plants, which urges the need for profound investigations in the marine environment.[5]

One of the better understood examples of an algae/pathogen interaction is the system of the red alga *Chondrus crispus* and its pathogen, the green alga *Acrochaete operculata*. The green alga is able to grow within the tissue of the red algal host. This process, called endophytism, can be fended off during certain developmental phases of *C. crispus*.[6] During the resistant gametophytic phase of the life cycle, *C. crispus* can recognise the attacker[6] and kill it by an immediate release of increased levels of H_2O_2 followed by the long-term up-regulation of defensive metabolites. In contrast, during the susceptible sporophytic phase, the green alga can penetrate the tissue of the host and parasitize it.[6]

Here we report the identification of the signal that triggers the early defense reaction of resistant gametophytes of the red alga. In addition, details are given about the biochemical processes that lead to the initial release of micromolar concentrations of

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