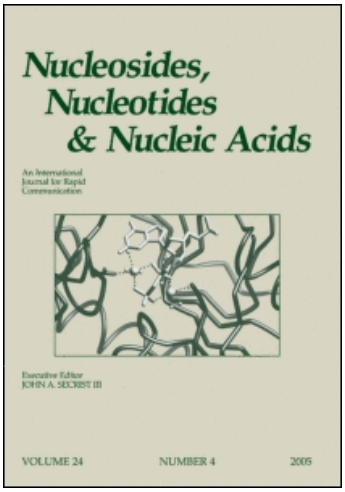

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Synthesis of (2',5')-Oligoadenylate Antisense Chimeras Targeting Steroid 5α-Reductase

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SYNTHESIS OF (2',5')-OLIGOADENYLATE ANTISENSE CHIMERAS TARGETING STEROID 5α-REDUCTASE

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Abstract. 2-5A antisense chimeras have been synthesized which target human steroid 5α-reductase mRNA. To enhance the stability of the chimera towards degradative enzymes the terminal phosphodiester bond was isomerized from 3',5' to 3',3' and the 5'-phosphate group was thiolated.

Introduction. The conjugation of a 5'-phosphorylated 2',5'-oligoadenylate (2-5A) *via* a linker to an oligonucleotide of a complementary sequence to a targeted sequence in an RNA (antisense oligonucleotide) provides a novel reagent for the selective and specific cleavage of RNA both in cell free systems and in intact cells[1,2].

In nature, 2-5A is generated from ATP by any of several isozymes of 2-5A synthetase, which are induced by treatment of cells with interferons and which are specifically activated by double-stranded RNA[3]. In the absence of 2-5A, the ubiquitous RNase L (2-5A-dependent RNase) is catalytically inactive[4]; however, nanomolar concentrations of 2-5A activate RNase L, resulting in the cleavage of single-stranded RNA.

Our strategy relies on the specific binding of the antisense oligonucleotide to a chosen RNA and subsequent activation of the RNase L which then specifically cleaves the targeted mRNA. Here the targeted mRNA encodes for the enzyme human steroid 5α-reductase type I.

5α-Reductase (5α-R) catalyzes the hydrogenation of testosterone to 5α-dihydrotestosterone (DHT). Two isoenzymes of 5α-reductase with clearly different characteristics have been described, 5α-reductase type I and type II. While 5α-reductase type II is mainly located in the prostate, the type I enzyme is predominant in the peripheral tissues, mainly the skin (hair follicles, sweat glands) and the liver. Excessive activity of 5α-reductase has been suggested as an etiologic factor in a variety of diseases including benign prostatic hypertrophy (BPH), prostatic carcinoma, acne, hirsutism and male-pattern baldness.

The human hair follicle offers a potentially very useful model for investigating androgen action. The effects of the various human endocrine abnormalities are readily visible and drugs can be applied as topical solutions.

Determination of the Sequence of an 2-5A-Antisense Oligonucleotide.

The nucleic acid sequence of the targeted mRNA has to be known: The sequence of the human steroid 5α-reductase (Type I) mRNA was determined by Andersson and Russell in 1990[5]. The identified cDNA contains 2102 bases, where the coding sequence (CDS) runs from base 31 to 810.

- Designed oligonucleotide has to be complementary to a portion of the mRNA.

- General considerations to the secondary structure of a mRNA: Single stranded RNA molecules have regions in which the polymer "folds back" and self hybridizes. These regions of self hybridizing duplex RNA are called "stems" and are separated by single-stranded "loops" and "bubbles". Thus, not all portions of the 5α-reductase mRNA are equally susceptible to the antisense oligonucleotide.

- Structure evaluation of a mRNA: Which portions of an RNA molecule are in stems and which are in loops or bubbles for the purposes of the intervention is determined by a computer modeling program such as MFOLD. Secondary structure prediction for RNA is fundamentally different from three dimensional molecular modeling[6]. A secondary structure of an RNA molecule is simply a collection of predicted base pairs subject to a few simple rules. Base pairs can be either G-C or A-U Watson-Crick pairs, or the weaker G-U pair. Negative stabilizing energies are assigned to the stacking of base pairs in helical regions, and to single bases that stack at the ends of helical regions. Otherwise, destabilizing energies are assigned to bulge, interior, hairpin and multi-branched loops. The energies of base stacking and the destabilizing loops are assumed to be additive in computing the overall energy.

MFOLD by Zuker and Jaeger systematically assesses all possible conformations and determines the conformation that is the most thermodynamically favored, i. e., has the lowest "free energy". Conformations that have a free energy within 5 or 10% of the optimal conformation are also determined. Most often these nearly optimal (suboptimal) foldings are closely related to each other. Thus, Zuker's MFOLD algorithm generates a group of plausible RNA secondary structures, which differ only slightly in energy, typically in increments of only 0.1 Kcal/mol.

- Considerations in selecting a suitable target:

• since the RNase L (2-5A-dependent. RNase) responsible for the action of 2-5A-antisense is active only on single-stranded sequences[4,7], it is important that there be stretches of non-base paired nucleotides near the chosen RNA target sequence.

• Since the RNase L prefers cleavage after UpN sequences[4,7], it is preferred that the single-tranded region where cleavage may occur should contain uridine. This is preferred but not essential as it has been shown with PKR RNA that 2-5A-antisense can cleave after other nucleotides[2].

• Since with PKR RNA as a target[8], cleavage occurs upstream (on the 5'-side) of the RNA target sequence, it is preferred that such uridine-containing single-stranded regions should be upstream of the target sequence.

• Since the antisense domain of the 2-5A-antisense chimera must form a double-helical complex with the RNA target sequence[1], it is preferable that such a targeted sequence be located in a single-stranded or at least partially single stranded region of the target RNA. This is due to the consideration that such a complex formation is an equilibrium process, and the magnitude of association constant for the process is reduced according to the degree and stability of the secondary structure within the specific target sequence.

• Because 2-5A-antisense operates catalytically[8], there must exist a necessary mechanism for the dissociation of the 2-5A-antisense chimera from its complementary sequence in the target RNA. Thus, it is to be expected that duplexes with a large number of GC base pairs would undergo dissociation with more difficulty than those with a high dA-rU or dT-rA content.

These preferences are not concrete. Since base-pairing and secondary structure is dynamic, some tolerance is expected in the amount of double-stranded structure acceptable in either antisense domain target or in the potential cleavage site.

Selecting the target sequences for 5α-reductase mRNA. Consideration of above leads to the search for the most preferred target sequence in the 5α-reductase mRNA. This target ideally should be single-stranded throughout the entire sequence that serves as the antisense binding site as well as the region upstream on the RNA of at least 16 nucleotides (based on the cleavage pattern of PKR RNA with 2-5A-antisense). Thus in the ideal situation the preferred target site should be the length of the antisense domain (e. g., 18) plus 16 equals 34 nucleotides in length.

The squiggle plot of the structure with the lowest energy of 5α-reductase shows the biggest loop at the end of the coding sequence at nucleotides 789-814, which is consistent in all suboptimal foldings. The single-stranded region is 25 nucleotides long and thus, does not have the minimum length for an ideally target which is 34. Nevertheless it was chosen as a prime target. The sequence at nucleotides 539-590 is a structure with only 6 base pairs on a stretch of 41 nucleotides, which is consistent in all suboptimal foldings, as well. For this reason it was chosen as another target. In a similar way other regions of the mRNA were chosen as targets for 2-5A-antisense chimeras.

Stability Enhancement of the chimera. The 2-5A antisense chimeras have the general structure p5'(A2'p)$_3$5'A2'p-(O(CH$_2$)$_4$Op)$_2$-5'N3'p)$_n$5'N where N represents any of the four common DNA nucleosides. To enhance the stability of the chimera towards degradative enzymes two structural variations were introduced. To make the antisense part more stable against exonucleases the terminal phosphodiester bond was simply isomerized from 3',5' to

3',3' to yield a capped structure. Second the 5'-phosphate group was thiolated. It has been shown that the 5'-thiophosphate analogue of 2-5A is highly resistant to enzymatic 5'-dephosphorylation and activates RNase L as effectively as p5'(A2'p)$_3$5'A itself[9].

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