Antiviral Research, 17 (1992) 279–287

AVR 00542

Mini-Symposium – HIV Infections*

Antisense inhibitors of HIV: problems and perspectives

Geneviève Degols, Jean-Paul Leonetti, Pierre Milhaud, Nadir Mechti and Bernard Lebleu

UA CNRS 1191 Génétique Moléculaire, U. Montpellier II Sciences et Techniques du Languedoc, Montpellier, France

Introduction: the SNAIGE concept

Pioneered by P. Zamecnik, P.O.P. Ts'O, D. Knorre and their colleagues, the use of synthetic oligonucleotides as a tool to control gene expression has been the object of an increasing number of studies from both academic and corporate laboratories. Several international meetings, a journal (Antisense Research and Development) and a dozen high-tech companies, reflect the growing interest of the scientific community.

As summarized in Table 1, synthetic oligonucleotides offer various possibilities of controlling gene expression; some have been barely explored.

Modifying gene expression by sequence-specific recognition of mRNA or pre-mRNA targets, with complementary oligonucleotides (generally ranging from 12- to 20-mer length), represents the antimessenger or antisense strategy strictly speaking. This strategy has been the most thoroughly investigated, as reviewed by Hélène and Toulmé (1991). Many cellular or viral genes have been targeted through this approach with a number of successes including phenotypic changes in the treated cells. The mechanisms governing these changes have, however, seldom be evaluated; potential sites for antisense oligonucleotides' action include interferences with pre-mRNA processing and nucleo-cytoplasmic transport, interferences with mRNA translation as well as mRNA degradation through RNase H activation.

Initial studies in cell-free systems and a few studies in intact cells have revealed the possibility of inserting a pyrimidine strand within DNA major

*Based on a presentation originally made at the Fourth International Conference on Antiviral Research held in New Orleans, Louisiana, U.S.A., April 21–26, 1991.

Correspondence to: G. Degols, UA CNRS 1191 Génétique Moléculaire, U. Montpellier II Sciences et Techniques du Languedoc, 34095 Montpellier, France.

TABLE I

Various possibilities for controlling gene expression with synthetic oligonucleotides

Synthetic oligonucleotides can be engineered to bind in a sequence-specific way:
(1) with complementary RNA through Watson-Crick pairing;
antisense oligonucleotides
(2) with duplex DNA in polypurine-polypyrimidine regions through Hoogsteen pairing;
triple-helix-forming oligonucleotides
(3) with proteins which constitute their already known targets (DNA binding proteins, viral polymerase binding sites, viral transactivators, ...) or even unknown targets (**aptamers**).

groove at the level of polypurine-polypyrimidine tract (see Nielsen, 1991, for a review). The formation of a local triplex structure involves base pairing through the rules proposed by Hoogsteen. This 'anti-gene' approach offers interesting possibilities of regulating gene expression through RNA polymerase inhibition, interferences with regulatory protein-DNA interactions (Maher et al., 1989) or even site-specific DNA alterations (François et al., 1989).

Increasing knowledge of the molecular basis of the interactions between regulatory proteins and their DNA (or RNA) binding sites allows the definition of oligonucleotides interfering specifically with nucleic acid-protein recognition, either by masking protein-binding sites or by titrating these proteins (Wu et al., 1990). It has not been explored to a large extent but deserves attention with particular regard to virus transactivation.

Synthetic oligonucleotides mimicking the catalytic properties of plant (e.g. the hammerhead structure) or animal (e.g. delta hepatitis catalytic motif) viral RNAs are the object of intensive studies. Sequence-specific cleavage of target RNA has been achieved in several tissues (e.g. Sarver et al., 1990) but the turnover rates of these artificial ribozymes are still far from being satisfactory.

Last but not least, progresses in nucleic acid chemistry and the advent of polymerase chain reaction amplification have paved the way for the innovative 'aptamer' concept (Tuerk and Gold, 1990). Briefly, it consists in selecting and amplifying from a collection of 10^{12} synthetic oligonucleotides the sequences exhibiting the appropriate affinity for a protein target, whether or not that particular protein was physiologically geared towards nucleic acid binding.

Besides natural oligonucleotides, numerous analogs with modified phosphodiester linkages or modified sugars, as well as 5′- or 3′-derived oligonucleotides, have been synthesized. They offer interesting possibilities with regard to metabolic stability, cell penetration or oligonucleotide-target interaction (see Goodchild, 1990, for a recent review).

Given such various modes of action, a synthetic oligonucleotide aiming to alter gene expression in a sequence-specific way cannot be adequately described by terms such as antimessenger, anti-gene or gene-blocker; SNAIGE, for

Synthetic Nucleic Acids Interfering with Gene Expression, might be more appropriate.

Problems in the use of synthetic oligonucleotides

Synthesizing oligonucleotides of the appropriate sequence has appeared as a rational and straightforward approach to designing highly specific gene expression modifiers, and in particular antiviral agents which are unfortunately scarce. Although these encouraging premises basically hold true, difficulties have been underevaluated giving rise to a flurry of ill-controlled data, of failures, as well as undisputed successes. These problems are summarized in Table 2. Synthesis and in vivo behavior will not be discussed; the former has been the object of several excellent reviews (Cohen, 1989) and the latter is as yet only scantly documented.

Target choice has turned out not to be as predictable as initially forecast for (1) a lack of knowledge about the three-dimensional structure of most RNA targets within their natural environment, (2) the near impossibility of predicting accessible nucleic acid sequences within ribonucleoprotein complexes or chromatin structure and (3) our as yet poor understanding of the rules governing sense-antisense or nucleic acid-protein interactions. Splice sites on pre-mRNAs, as well as 5′-untranslated regions on mRNAs generally appear as the most efficient targets in the antisense approach, but exceptions have been documented.

Metabolic stability is low due to the action of nucleases (mainly 3′-exonucleases) in extracellular fluids, endocytic compartments and intracellular environment. Various analogs modified at either the internucleotidic phosphate backbone (e.g. methylphosphonates or phosphorothioates), the sugar configuration (e.g. α-oligonucleotides or 2′-O methyl oligonucleotides) or the 3′-end have been synthesized and provide adequate solutions to this particular problem.

Reconciling these modifications which lower nuclease sensitivity with the

TABLE 2

Problems in the use of synthetic oligonucleotides as potential therapeutic agents

- In vivo and in vitro distribution;
- metabolic stability;
- target choice and structure;
- processing and stability of target-oligonucleotide complexes;
- toxicology and immunogenicity;
- large-scale production and manufacturing costs.

structural features required for sequence-specific nucleic acid recognition at useful T_m, and eventual processing of the hybrids by RNase H has, however, proven to be more difficult than initially anticipated. Moreover, little is still known about the toxicity of the metabolites arising from oligonucleotide analogs' degradation products. An alternative approach involves the association of unprotected oligonucleotides to drug delivery systems such as liposomes, lipoproteins particles, nanoparticles or protein conjugates, as developed initially by our group (vide infra).

Another problem met with the use of synthetic oligonucleotides deals with cell uptake and intracellular compartmentalization. Oligonucleotides are now believed to be taken up by cells through receptor-mediated endocytosis after binding to cell surface proteins (Loke et al., 1989; Yakubov et al., 1989); several candidate receptors have been recently described and partially characterized. Conjugation to synthetic polypeptides as poly(L-lysine) (Lemaitre et al., 1987), to lipids as cholesterol (Letsinger et al., 1989), or encapsidation in antibody-targeted liposomes (Leonetti et al., 1990) increases cell uptake and biological efficacy, strongly suggesting cell uptake of unmodified oligonucleotides as a limiting step; this point will be detailed later on.

Whether oligonucleotides are used in their free form, or in association with the delivery systems briefly mentioned above, their internalization involves receptor-mediated (or fluid-phase) endocytosis. Escaping the endocytic compartments to reach intracellular targets in the cytoplasm or in the nucleus is another problem we have to cope with.

Trapping of oligonucleotides within endocytic compartments and/or degradation by lysosomal nucleases might well represent a strong limitation in this approach. Neutral analogs as methylphosphonates should in principle bypass these steps (Loke et al., 1989); however, they act in the 10–100 μM range in the various biological models described so far, suggesting additional limitations in their use. Another attractive prospect makes use of fusogenic or pH-sensitive liposomes whose encapsidated content should be released in cell cytoplasm through fusion at neutral pH with the plasma membrane or at slightly acidic pH at the endosome level. No data have yet been obtained with antisense oligonucleotides to our knowledge; efficient functional delivery of genes (see Wright and Huang, 1989, for a review) or more recently of dsRNAs (as monitored by IFN and IL6 induction) has been achieved (Milhaud et al., submitted for publication).

An unexpected feature of oligonucleotides' intracellular behavior has arisen from microinjection studies, indicating their rapid diffusion to the nuclei; they bind to a set of nuclear proteins of yet unknown nature (Leonetti et al., 1991). Whether this favors the interaction of synthetic oligonucleotides with nuclear targets or segregates oligonucleotides in the nuclei is not known.

The fate of oligonucleotides-target hybrids represents an additional ill-understood event with relevance to biological efficacy. Physical association through hybridization could lead to the activation of endogenous RNase H with an expected increment in inhibitory activity (Walder and Walder, 1988).

TABLE 3

Synthetic oligonucleotides as anti-HIV agents

• Sequence-specific and dose-dependent inhibitions with EC_{50} around 5 μM for phosphorothioate derivatives, in chronically-infected cells.
• Little or no sequence-specificity in de novo infected cells. The most active S-dC28 compound has an EC_{50} of 0.5 μM; it probably acts as a template primer competitive inhibitor at the level of HIV reverse transcriptase and as a competitor for virus/cell receptor interaction.
• Synthetic 'hammerhead' ribozymes were designed to promote sequence-specific cleavage of HIV *gag* protein mRNA.
• Polypurine-polypyrimidine tracts are available for triple helix formation at several locations in HIV genome.
• Increased molecular knowledge of RRE-*rev* or TAR-*tat* interactions will allow the design of appropriate antisense or competitor oligonucleotides.

Whether and to what extent RNase H actually potentiates antisense oligonucleotide activity in somatic cells has not been formally demonstrated although fair evidence for such a mechanism has been raised in *Xenopus laevis* oocytes (Dash et al., 1987).

A new generation of oligonucleotides engineered to destroy their target RNA (or DNA) or to bind covalently to them is now being studied in many laboratories. It includes oligonucleotides linked to alkylating moieties (Knorre et al., 1985), free-radical generating groups (Boutorin et al., 1984; Chen and Sigman, 1986) or photoactivable ones (Shi and Hearst, 1986). Oligonucleotides conjugated to intercalating drugs with the aim of increasing binding constant should also be mentioned (Hélène et al., 1985).

At the opposite, cells are equipped with a collection of RNA secondary structures' unwinding activities. Their impact on the efficacy of antisense oligonucleotides has not been evaluated except for in *Xenopus laevis* embryos where they seriously impair the usefulness of this approach to studying development (Rebagliati and Melton, 1987).

Synthetic oligonucleotides in the control of HIV expression: a brief overview

The sophisticated mechanism(s) set up by HIV to replicate in T lymphocytes or in macrophages have been reviewed on many occasions. Attempts to control HIV expression by various synthetic oligonucleotides have been numerous. Reverse transcription and the production of the *rev* or *tat* transactivating proteins have been the most often used targets; Table 3 schematizes available data in this regard.

In de-novo-infected T lymphocyte cell lines, oligonucleotides with various targets and modifications have been used. They turned out to be poorly specific in term of sequence recognition. The most efficient and best studied one is a

TABLE 4

Antiviral activity of *tat* oligonucleotide-PLL conjugates in cells infected with different HIV isolates

Virus isolate	Genomic sequence at oligonucleotide target	*tat*-PLL conjugate	RT activity (cpm/ml)	% RT inhibition
HIV-1 BRU	5′-AGCCAGTAGATCCTAG-3′ (5416–5431)	–	32 500	–
		+	2 300	93
HIV-1 ELI	5′-ATCCAGTAGATCCTAA-3′ (5380–5395)	–	21 200	–
		+	15 400	27
HIV-2 ROD	5′-AGACACCCTTGAAGGC-3′ (5449–5464)	–	10 000	–
		+	12 200	–

MT4 cells were infected at 4°C with the virus concentration of each isolate. After a 30-min adsorption time, cells were washed, transferred to 37°C and incubated with 0.5 μM anti-*tat* BRU PLL conjugates. Reverse transcriptase activities were measured 5 days post-infection.

(dC)28 homopolymer with phosphorothiorate internucleotidic linkages which exhibits an EC_{50} around 0.5 μM (Matsukura et al., 1987). Non-sequence-specific biological activity probably results from competitive inhibition for substrate binding on viral reverse transcriptase (Majumdar et al., 1989) or from interferences with virus adsorption to its CD4 membrane receptor. Let us also cite phosphorodithioate oligonucleotide derivatives which appeared rather

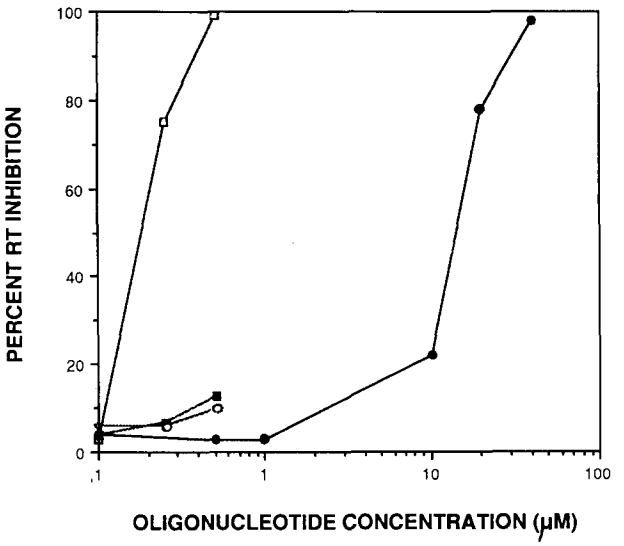

Fig. 1. Inhibition of HIV-1 expression by unconjugated and PLL-conjugated *tat* antisense oligomer. MT4 cells were infected with an equal volume of virus-containing medium (1000 $TCID_{50}$) and incubated for 30 min at 4°C. Cells were washed and incubated at 37°C with the indicated concentration of oligonucleotides or PLL conjugates. Inhibition of HIV expression was assayed by reverse transcriptase activity 4 days post-infection. Anti-*tat* oligonucleotide: ●; PLL: ■; random oligonucleotide PLL: ○; anti-*tat* oligonucleotide PLL: □.

potent in cell-free studies (M. Caruthers, personal communication) or cholesteryl derivatives with an EC_{50} = 0.2 μM. (Letsinger et al., 1989); in neither case was sequence specificity revealed. We have initiated a comparative study of 12-mer oligonucleotides complementary to *tat* splice acceptor site in collaboration with the group of Prof. J.L. Imbach (Lab. Chimie Bio-Organique, U. Montpellier II); our results go along the same lines with little or no sequence specificity and the following order of efficiency: αS or βS > > α or Met P > β.

We have used the same biological model, e.g. de novo infection of MT4 lymphocytes by HIV-1 BRU (1000 $TCID_{50}$), to investigate the antiviral activity of antisense oligonucleotides complementary to the translation initiation region of *tat* mRNA (nucleotides 5416 to 5431 in the genome of HIV-1BRU). The 16-mer oligonucleotides (anti-*tat*) had a phosphodiester backbone and carried a 3'-riboadenosine residue for further coupling to ε-aminogroups of poly(L-lysine) (PLL) as previously described (Leonetti et al., 1988). Oligonucleotides or oligonucleotide PLL conjugates were administered to MT4 lymphocytes growing in regular conditions (RPMI 1640 medium complemented with 10% (v/v) fetal calf serum) as a single addition after virus adsorption. Antiviral activities were monitored through conventional reverse transcriptase (Fig. 1), syncytia formation or T cell viability assays; similar conclusions were reached from all three assays (not shown). As illustrated in Fig. 1, anti-*tat* oligonucleotide PLL conjugates are much more active (EC_{50} = 0.2 μM) than non-conjugated material (EC_{50} = 20 μM), the latter value being comparable with published data for natural oligonucleotides (Goodchild et al., 1988). Neither PLL alone nor random oligonucleotide PLL conjugates (e.g. oligonucleotides with the same base composition but no sequence relatedness) exhibit appreciable antiviral activity in these assays. The genetic variability of HIV viruses allowed us to confirm sequence-specific recognition by oligonucleotide-PLL conjugates. The same anti-*tat* oligonucleotide PLL conjugate was tested against the HIV-1 BRU strain with which perfect matching is realized as well as against HIV-1 ELI (two mismatches in the oligonucleotide target region) or against HIV-2 (which has little sequence homology in the oligonucleotide target sequence). As shown in Table 4, the antiviral activity of the anti-*tat* oligonucleotide PLL conjugates parallels the extent of matching with the target sequence as expected for sequence-specific recognition. Whether the use of oligonucleotides with a regular phosphodiester backbone at low concentration or other unknown features of these oligonucleotide PLL conjugates is responsible for these data should be further evaluated. Whatever the case it offers an interesting tool for the screening of the most efficient oligonucleotide sequences and/or oligonucleotide modifications. The drawback is of course that clinical HIV isolates contain many variants for which efficient and conserved regions will have to be sought.

Sequence specificity has on the contrary generally be attained in chronically-infected T lymphocytes. The most active and best studied compound is a 27-mer phosphorothioate analog complementary to the 5'-end of rev mRNA; it

has an EC_{50} around 5 μM (Matsukura et al., 1989). Oligonucleotides with phosphodiester backbones have EC_{50} above 25 μM in similar assays.

The *ribozyme* approach has been the object of intense NIH-fostered research in several laboratories. As an example, hammerhead catalytic motifs targeted to HIV *gag* protein mRNA have been expressed in transfected cells. Synthetic oligonucleotides against the same region or composite structures harboring RNA sequences in the catalytic moiety and DNA (or DNA analogs) in the flanking target recognition regions have been synthesized and shown to be active. These constructions have to our knowledge not yet been successfully internalized in intact cells and tend to act more in a stoichiometric than catalytic way.

Rapid progresses in the molecular analysis of TAR-*tat* and RRE-*rev* interaction are being made; the potential antiviral activity of oligonucleotides mimicking (or complementary to) the active regions has not yet been tested to our knowledge.

Several regions on the HIV genome are putatively able to form triple helices but no experimental data have yet been disclosed.

Conclusion and perspectives

Synthetic oligonucleotides offer exciting prospects as tools for molecular analysis of HIV replication and as possible specific antiviral agents. Much has yet to be learned about their mechanism of cell uptake, intracellular distribution and mechanism of action (to say nothing of their biodisposability, immunogenicity and toxicity). The unforeseen difficulties encountered in their use should not lead to scepticism but on the contrary be a source of optimism. It is indeed our personal feeling that the presently available oligonucleotides and analogs will greatly gain in efficacy and specificity when properly formulated.

References

Boutorin, A.S., Vlassov, V.V., Kazakov, S.A., Kutiavin, I.V. and Podymnogin, M.A. (1984) Complementary addressed reagents carrying EDTA-Fe(II) groups for directed cleavage of single-stranded nucleic acids. FEBS Lett. 172, 43–46.

Chen, C.H.B. and Sigman, D.S. (1986) Nuclease activity of 1.10-phenantroline-copper: sequence-specific targetting. Proc. Natl. Acad. Sci. USA 83, 7147–7151.

Cohen, J.S. (1989) Oligodeoxynucleotides. Antisense inhibitors of gene expression, Macmillan Press, London.

Dash, P., Lotan, I., Knapp, M., Kandel, E.R. and Goelet, P. (1987) Selective elimination of mRNAs in vivo: complementary oligodeoxynucleotides promote RNA degradation by an RNase H-like activity. Proc. Natl. Acad. Sci. USA 84, 7896–7900.

François, J.C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N.T. and Hélène, C. (1989) Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate. Proc. Natl. Acad. Sci. USA 86, 9702–9706.

Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. Bioconjugate Chem. 11, 165–187.

Goodchild, J., Agrawal, S., Civeira, M.P., Sarin, P.S., Sun, D. and Zamecnik, P.C. (1988) Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA 85, 5507–5511.

Hélène, C., Montenay-Garestier, T., Saison, M., Toulmé, J.J., Asseline, U., Lancelot, G., Maurizot, J.C., Toule, F. and Thuong, N.T. (1985) Oligodeoxyribonucleotides covalently linked to intercaling agents: a new class of gene regulatory substances. Biochimie 67, 777–783.

Hélène, C. and Toulmé, J.J. (1990) Specific regulation of gene expression by antisense, sense or antigene nucleic acids. Biochem. Biophys. Acta 10491, 99–125.

Knorre, V., Vlassov, V.V. and Zarytova, V.F. (1985) Reactive oligonucleotide derivatives and sequence-specific modification of nucleic acids. Biochimie 67, 785–789.

Lemaitre, M., Bayard, B. and Lebleu, B. (1987) Specific antiviral activity of a poly(L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site. Proc. Natl. Acad. Sci. USA 84, 648–652.

Leonetti, J.P., Mechti, N., Degols, G., Gagnor, C. and Lebleu, B. (1991) Intracellular distribution of microinjected antisense oligonucleotides. Proc. Natl. Acad. Sci. USA 88, 2702–2706.

Leonetti, J.P., Machy, P., Degols, G., Lebleu, B. and Leserman, L. (1990) Antibody-targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication. Proc. Natl. Acad. Sci. USA 87, 2448–2451.

Letsinger, R.L., Zhang, G., Sun, D.K., Ikeuchi, T. and Sarin, P.S. (1989) Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. Proc. Natl. Acad. Sci. USA 86, 6553–6556.

Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S. and Nekers, L.M. (1989) Characterisation of oligonucleotide transport into living cells. Proc. Natl. Acad. Sci. USA 86, 38474–38478.

Maher, III, L.J. (1989) Inhibition of DNA binding proteins by oligonucleotide-directed triple-helix formation. Science 245, 725–730.

Majumdar, C., Stein, C.A., Cohen, J.S., Broder, S. and Wilson, S.H. (1988) Stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide. Biochemistry 28, 1340–1346.

Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J.S. and Broder, S. (1987) Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 84, 7706–7710.

Matsukura, M., Zon, G., Shinozuka, K., Robert-Gurrof, M., Shimada, T., Stein, C.A., Mitsuya, H., Wong-Stall, F., Cohen, J.S. and Broder, S. (1989) Regulation of viral expression of human immunodeficiency virus in vitro by an antisense phosphorothioate oligodeoxynucleotides against rev (art/trs) in chronically infected cells. Proc. Natl. Acad. Sci. USA 86, 4244–4248.

Nielsen, P.E. (1991) Sequence-selective DNA recognition by synthetic ligands. Bioconjugate Chem. 24, 1–12.

Sarver, N., Cantin, E.M., Chang, P.S., Zaia, J.A., Ladne, P.A., Stephens, D.A. and Rossi, J.J. (1990) Ribozymes as potential anti-HIV-1 therapeutic agents. Science 247, 1222–1225.

Rebagliati, M.R. and Melton, D.A. (1987) Antisense RNA injections in fertilized frog eggs reveal an RNA duplex unwinding activity. Cell 48, 599–605.

Rosen, C.G. (1991) Regulation of HIV expression by RNA-protein interactions. TIG 7, 9–14.

Shi, Y.B. and Hearst, J.E. (1986) Thermostability of double-stranded deoxyribonucleic acids: effects of covalent additions of a psoralen. Biochemistry 25, 5895–5902.

Tuerk, C. and Gold, L. (1990) Sciences 250, 1149–1151.

Walder, R.Y. and Walder, J.A. (1988) Role of RNase H in hybrid arrested translation by antisense oligonucleotides. Proc. Natl. Sci. USA 8501, 5011–5015.

Wright, S. and Huang, L. Antibody-directed liposomes as drug-delivery vehicles. Adv. Drug Deliv. Rev. 34, 343–389.

Wu, H., Holcenberg, J.S., Tomich, J., Chen, J., Jones, P.A., Huang, S.H. and Calame, K.L. (1990) Inhibition of in vitro transcription by specific double-stranded oligodeoxyribonucleotides. Gene 34934, 203–209.

Yakubov, L.A., Deeva, E.A., Zarytova, V.F., Ivanova, E., Ryte, M., Yurchenko, L.V. and Vlassov, V.V. (1989) Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? Proc. Natl. Acad. Sci. USA 86, 6454–6458.