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Plasma protein binding, lipoprotein distribution and uptake of free and lipid-associated BCL-2 antisense oligodeoxynucleotides (G3139) in human melanoma cells

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Abstract

The objectives of this study were to determine the protein binding and lipoprotein distribution of G3139 and G3139 lipoplexes following incubation in human plasma, assess complement activation of, and the effect of pre-incubation of G3139 and G3139 lipoplexes in human plasma on in vitro cellular uptake of G3139. Effect of concentration and time on incorporation of free and lipid associated (lipoplexes) [3H]*Bcl-2* AO (25–600 ng/ml) into normolipidemic human plasma lipoproteins was determined by density gradient ultracentrifugation after incubation at 37 °C for 5, 30, 60 and 120 min. Protein binding in the lipoprotein deficient fractions (LPDP) was determined by equilibrium dialysis. Complement interaction was determined by ELISA after exposure of human plasma to AO +/− liposomes prepared in serial dilution. In vitro uptake of G3139 and G3139 lipoplexes into human melanoma cells was assessed qualitatively by fluorescence microscopy after 4-h exposure to G3139 (free or as lipoplexes) with or without pre-incubation of G3139 in normal human plasma. Analysis of Bcl-2 AO-lipoprotein interaction over time and concentration indicated no significant movement of the compound within the different lipoprotein and LPDP fractions. Majority of drug was recovered within LPDP fraction, and more than 85% of drug recovered within LPDP fraction was protein bound. No significant activation of complement was noted for either free AO or lipoplexes. Pre-incubation of free AO or AO-lipoplexes in human plasma resulted in a greater cellular uptake of AO-lipoplexes compared with plasma free controls. These findings suggest that the majority of [3H]Bcl-2 AO is plasma protein bound with little lipoprotein association and no significant movement between different lipoprotein and LPDP fractions. Plasma protein binding other than lipoprotein binding may be responsible for the difference in cellular uptake of free AO vs. cationic lipoplexes.

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1. Introduction

Antisense therapy has come to the forefront as a novel modality for the treatment of cancer and a variety of other human diseases. Phosphorothioate-modified oligodeoxynucleotides (ODN) have been especially promising, with improved stability and longer half-life than phosphodiester-type ODN. In humans, ODN pharmacokinetics generally fit a two-compartment model, with a rapid initial plasma elimination rate (10–30 min) followed by a longer terminal half-life (24–48 h) Wallace et al., 1998. ODN appear to be effective in treatment of preclinical models. Various antisense therapies are being developed for clinical applications using aqueous soluble formulations of ODN. However, in vitro activity of ODN generally requires the use of cationic liposomes or other delivery systems. This discrepancy between in vitro and in vivo activity of free ODN raises the question of what mediates antisense oligonucleotide disposition and cellular uptake in vivo, which we are beginning to address in this study. We propose that specific components of the bloodstream, such as plasma proteins (albumin, α-1-glycoprotein), lipoproteins and complement proteins, that are found in greater concentrations in vivo than in vitro (e.g. within tissue culture medium) may play a role in the disposition of ODN. Many studies have shown that ODN can bind specific protein Agrawal, 1998. Serum albumin has been demonstrated to be a major binding protein of oligonucleotides Agrawal et al., 1998, yet it is not clear whether binding to serum albumin is important for ODN activity. It remains to be determined what other specific proteins in the blood bind to phosphorothioate oligonucleotides. It will also be interesting to know if plasma protein binding directly influences the cellular uptake of ODN in vivo.

The objectives of this study were: (1) to determine if human plasma or serum would alter the cellular association of free ODN and liposome-associated (lipoplex) ODN with human cells in vitro; (2) to determine the plasma protein binding and lipoprotein distribution of a therapeutically active phosphorothioate ODN, an antisense targeted against the anti-apoptosis gene product, (BCL2) (referred to as G3139); and (3) to determine if free G3139 and lipoplex G3139 activate complement in human plasma. The antisense molecule G3139 can down-regulate BCL2 expression resulting in reduced cell viability and increased chemosensitivity due to its role in modulating apoptosis Cotter et al., 1994. Multiple clinical trials using G3139 and chemotherapy are presently ongoing in North America and Europe for treatment of melanoma, lymphoma and other cancers Jansen et al., 2000; Waters et al., 2000.

As noted above, experimental systems utilizing tissue culture models often require lipofection or other means to deliver the antisense molecules to the cells. Attempts have also been made to utilize delivery systems to increase in vivo circulation lifetime of ODN such as with lipid-based or particulate formulations Garcia-Chaumont et al., 2000. Several studies have investigated the effect of liposome association of ODN on their subsequent pattern of uptake, cellular processing and antisense activity to determine if this technology may be beneficial Bennett et al., 1992. It is clear that liposome association results in an alteration in the mechanism of cellular uptake compared with free oligonucleotides, but this effect is highly dependent on the formulation. Altered patterns of in vivo biodistribution have also been previously observed Bennett et al., 1996; Litzinger et al., 1996. In vitro, liposome association is thought to facilitate ODN release from the degradative endocytic compartment and thereby increase the likelihood of the ODN reaching its main site of action, the cytoplasm. However, formulations that are optimal for in vitro applications are often highly charged and fusogenic, making them unsuitable for in vivo systemic use. Regardless of the ODN formulation, the role of serum protein binding on the activity of the ODN (and its carrier if present) are poorly understood.

The present study investigates the differential interaction of phosphorothioate oligonucleotides, in free form or as lipoplexes, with plasma proteins, and the effect of that interaction on in vitro cellular uptake. Human melanoma cells were incubated with G3139 and G3139-lipoplexes, with or without pre-incubation of the ODN in human plasma or serum. Total protein binding and lipoprotein binding of G3139 in human plasma were also analyzed. G3139 was mixed with human plasma, lipoprotein fractions were separated and the amount of oligonucleotide was determined in each fraction. Complement activation assays were performed with G3139 and G3139 lipoplexes to determine if serum complement proteins had the potential to play a role in their disposition.

2. Materials and methods

2.1. Chemicals and plasma

G3139, a fully phosphorothioated, linear, single-stranded 18mer oligodeoxynucleotide [sequence: 5′-tct ccc agc gtg cgc cat-3′ $MW = 5764$] complementary to the first six codons of BCL2 mRNA as well at its tritiated form ([^{3}H]G3139, specific activity $= 0.846$ mCi/μmol), and FITC-labeled G3139 were obtained from Genta Inc. (Lexington, MA). Normolipidemic human plasma was obtained from Canadian Blood Services (Vancouver, British Columbia). Cholesterol, triglyceride and protein enzymatic assay kits were purchased from Sigma Chemical (St. Louis, MO).

2.2. In vitro uptake into human melanoma cells

In vitro uptake of FITC-labeled G3139 into 518A2 human melanoma cells was assessed after 4 h exposure to G3139 (free or lipoplexes) with or without 1 min pre-incubation of G3139 in normal human citrate-treated plasma or normal human serum. Controls consisted of no treatment or liposomes only ($+/-$ plasma or serum) ($n = 3$ for all treatment groups and controls). Liposomes composed of dioleyldimethylammonium chloride/dioleoyl phosphatidylethanolamine (DODAC/DOPE 1:1) were prepared by extrusion (mean diameter ~ 100 nm) and concentration determined by phosphorus assay. To prepare the cationic lipoplexes, G3139 was mixed with liposomes (25.7 μM ODN, 1.3 mM lipid) and incubated for 30 min at ambient temperature prior to use. The cells were washed four times with 1% bovine serum albumin in phosphate-buffered saline (pH 7.0) after the 4 h exposure to FITC-G3139, then methanol-fixed, mounted and photographed the same day. Samples shown are representative of three wells receiving the same treatment.

2.3. Plasma lipoprotein distribution studies

[^{3}H]G3139 at 25, 50, 100 and 200 ng/ml was incubated in normolipidemic human plasma (total cholesterol 100–160 mg/dl; total triglyceride 100–140 mg/dl) for 5, 30, 60 and 120 min at 37 °C. Four different batches of pooled human plasma were used. The plasma was separated into its HDL, LDL, triglyceride rich-lipoprotein (TRL) which includes very low-density lipoproteins and chylomicrons as well as lipoprotein-deficient lipoprotein plasma (LPDP; which includes albumin and α-1 glycoprotein) fractions by step-gradient ultracentrifugation as previously described Wasan et al., 1999. Each fraction was assayed for [^{3}H]G3139 by radioactivity. Recovery was determined using an external calibration curve. To assure that the distribution of [^{3}H]G3139 found in each of these fractions was a result only of its association with each lipoprotein or lipoprotein deficient fraction and not a result of its own density, [^{3}H]G3139 was incubated in LPDP fraction for 5 min at 37 °C and the density determined by ultracentrifugation. The majority of free [^{3}H]G3139 ($> 95\%$) was found in the range of 1.006–1.013 g/ml (data not shown) suggesting that the ODN distribution within the ultracentrifuge tubes following incubation in human plasma was not a function of ODN density itself.

2.4. Determination of ODN free fraction

To determine the unbound fraction of ODN, plasma containing [^{3}H]G3139 was separated into its lipoprotein fractions (HDL, LDL and TRL)

and LPDP fraction by density gradient ultracentrifugation. The percent protein bound of the LPDP fraction was determined using an ultrafuge filter column (Centrifree Micropartition Column; Amicon Inc., Beverly MA). Samples were centrifuged at RT at 4500 rpm (10 000 × g) for 2 h. The resulting filtrate obtained was analyzed for protein (modified Lowry assay kit, Sigma) and [^{3}H]G3139 (by specific activity). Greater than 99% of the protein was retained by this filter and therefore [^{3}H]G3139 in the filtrate represents free (unbound) drug. Nonspecific binding of [^{3}H]G3139 to the filter was 5–8%. Equilibrium dialysis was the second method to determine protein binding of ODN in human plasma. [^{3}H]G3139 (1000 ng/ml) was incubated in human plasma for 5 min at 37 °C. Following incubation the plasma was cooled to 4 °C and 1 ml was placed in a polycarbonate dialysis bag (MWCO 12 000–14 000, SpectroPor, Mississauga, ON, Canada) and dialyzed against 20 ml of 0.9% NaCl for 20–24 h. The percentage of ODN recovered within the dialysis tubing represented the percent of total protein binding. Nonspecific retention of ODN was $< 2\%$.

2.5. Human complement assay

Complement interaction was determined using normal human EDTA-treated plasma using a C3a enzyme immunoassay kit from Quidel (San Diego, CA). G3139 (1,4 and 10 μg/ml) in free form or as lipoplexes (charge ratio $(+/-) = 3$) was pre-incubated in human plasma for 1 min at 37 °C immediately prior to assay for C3a. Lipoplexes were prepared as described above. Enzyme assays were performed in triplicate and compared with a standard curve for C3a quantification. This assay reports the total C3a and C3a des arg (inactivated form) present in the sample. C3a is generated through both the classical and alternate pathways of complement activation.

2.6. Statistical analysis

Differences in antisense oligonucleotide distribution within plasma lipoproteins, plasma protein binding and differences in human complement activation in the presence of different concentrations of free ODN or ODN lipoplexes were analyzed by one-way analysis of variance (INSTAT2; GraphPad). Critical differences were assessed by Neuman–Keuls posthoc tests. Differences were considered significant if $P < 0.05$. All data are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. In vitro uptake of phosphorothioate oligonucleotides: effect of pre-incubation in human plasma and serum

Cellular uptake of G3139 was enhanced in 518A2 cells by liposome association (Fig. 1A). Pre-incubation of ODN or ODN-lipoplexes in human plasma (Fig. 1B) or serum (Fig. 1C) prior to addition to tissue culture medium increased the relative proportion of free G3139 or that of G3139 cationic lipoplexes associated with the cells. Without pre-exposure of free G3139 to plasma or serum, no cellular association was observed. The increase after pre-incubation was more marked for serum than plasma for free G3139 but similar for G3139 cationic lipoplexes on qualitative observation of fluorescence intensity. Changes in the pattern of uptake (i.e. from punctate fluorescence to a more diffuse pattern using lipoplexes) were observed in some samples but not all. It is not known if the ODN associated with the cells was internalized or adsorbed onto the surface, although an extensive washing procedure coupled with a punctate fluorescent pattern is consistent with endocytosis. Furthermore, these observations are consistent with quantitative in vitro data previously published Chi et al., 2000; Zhu et al., 2001.

3.2. Total protein and lipoprotein distribution of oligonucleotides

Regardless of concentration, the majority of [^{3}H]G3139 was recovered within LPDP fraction (Table 1). Incubation time also did not effect the incorporation of the ODN into the lipoproteins. When [^{3}H]G3139 (25–200 ng/ml) was incubated

in normolipidemic human plasmas for 5–120 min, 0.3–1.8% of the original concentration of [^{3}H]G3139 incubated was recovered in the triglyceride-rich lipoprotein fraction (containing chylomicrons and very low-density lipoproteins), 0.4–3.3% was recovered in the LDL fraction, 1.5–4.3% was recovered in the HDL fraction and 72.8–96.6% was recovered in the LPDP fraction (containing predominantly albumin and α-1 glycoprotein). Of the [^{3}H]G3139 recovered in the LPDP fraction $>85\%$ was protein bound. When lipoplex [^{3}H]G3139 was used, total protein binding was $\sim 98\%$. Total recovery ranged between 84–99%. These findings suggest that the majority of free [^{3}H]G3139 is plasma protein bound with little lipoprotein association and no significant transfer between different lipoprotein and LPDP fractions. However, with liposome-associated [^{3}H]G3139 a significantly greater percentage was found in the lipoprotein fraction, with the greatest

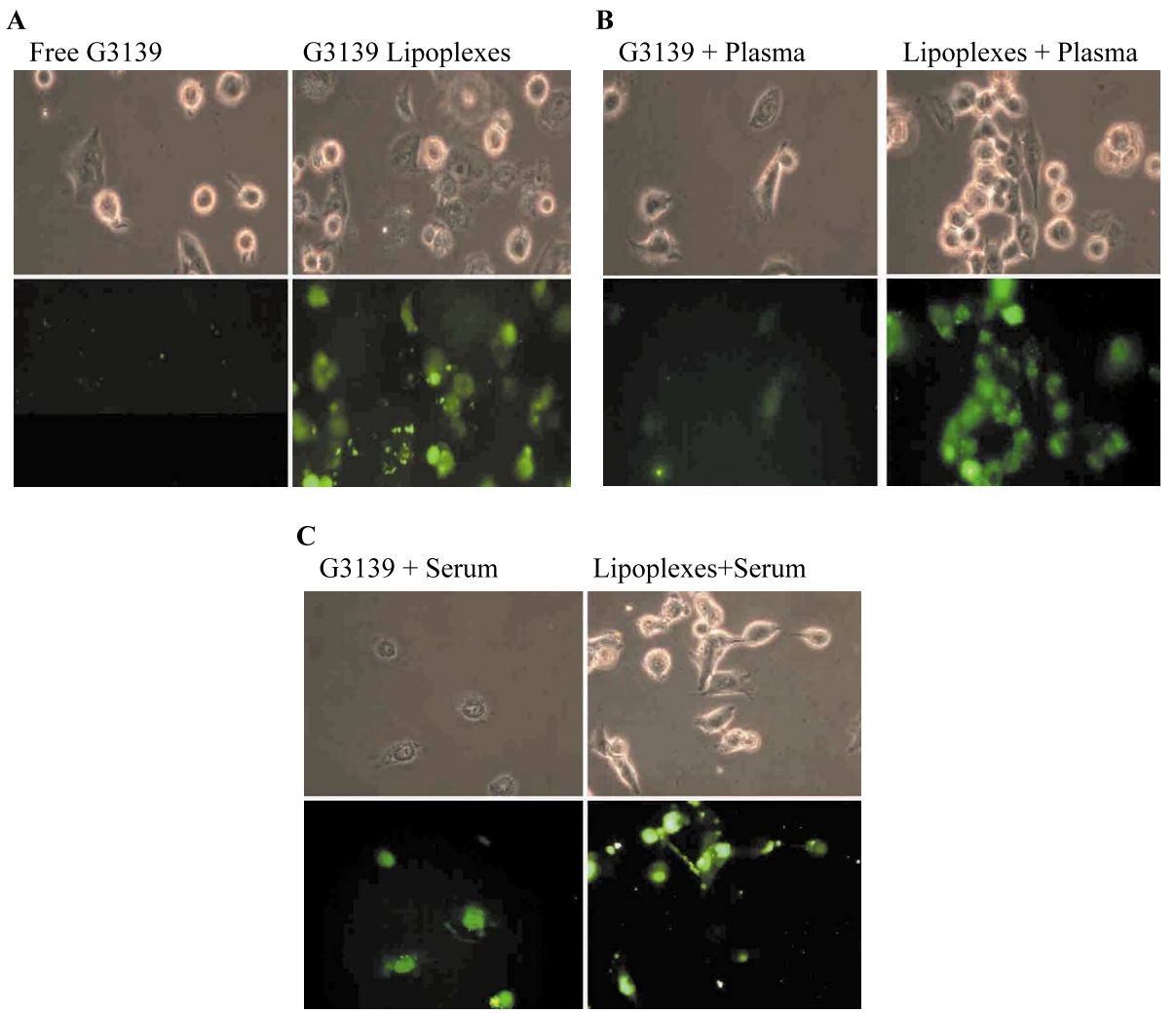

Fig. 1. Association of BCL-2 antisense oligodeoxynucleotides G3139 with 518A2 human melanoma cells is enhanced by DODAC/DOPE cationic lipoplexes (A) and by pre-incubation of free G3139 or G3139 lipoplexes in human plasma (B) or human serum (C) prior to exposure to cells. Phase contrast (top) and fluorescence (FITC) (bottom) are representative photomicrographs after 4 h incubation with G3139.

Table 1
Plasma distribution of [^{3}H]BCl2-antisense (oligodeoxynucleotide) following incubation of free (control) and lipid-associated (lipoplexes) G3139 in human plasma for 1 h at 37 °C

Treatment Group	Concentration (ng/ml)	TRL (%)[a]	LDL (%)	HDL (%)	LPDP (%)	Percent Recovery (%)[b]
Control	120	ND	ND	ND	96.1 ± 3.6	96.1 ± 3.6
Lipoplexes	120	2.6 ± 0.3	3.2 ± 0.2	4.5 ± 0.6	88.2 ± 6.9	98.5 ± 6.8
Control	600	ND	ND	2.8 ± 0.9	93.2 ± 3.3	95.9 ± 3.8
Lipoplexes	600	2.7 ± 0.3	5.1 ± 0.6	7.8 ± 0.3*	77.7 ± 3.7*	93.3 ± 3.8

Data is presented as mean ± standard deviation (S.D.) ($n = 3$); Abbreviations: TRL, triglyceride rich lipoproteins, which includes very low-density lipoproteins and chylomicrons; LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein deficient fraction, which includes albumin and α-1 glycoprotein; ND, non-detectable, below the limit of detection.
[a] percent of [^{3}H]BCl2-antisense (oligodeoxynucleotide) recovered in each fraction.
[b] percent of [^{3}H]BCl2-antisense (oligodeoxynucleotide) incubated in human plasma.
*$P < 0.05$ vs. Control.

proportion of that fraction associated with HDL. The percentage in the LPDP fraction also decreased when lipoplex concentration was greater (100 vs. 600 ng ODN/ml), increasing the percentage in both the HDL and LDL fractions by approximately 60%. The significance of these findings is not presently clear.

3.3. Complement activation

When free G3139 and G3139-lipoplexes were pre-incubated in human plasma, complement protein (C3a) activation was observed at 1, 4 and 10 μg/ml (Fig. 2). Free ODN generated significantly more C3a than the cationic lipoplexes at 1 and 4 μg/ml, but not at 10 μg/ml. DODAC/DOPE liposomes alone activated complement less than free ODN but to a similar level as the lipoplexes.

4. Discussion

Upon administration of the antisense oligonucleotide molecules into the bloodstream, which is a complex matrix of cells, proteins and other elements, ODN are rapidly cleared and either delivered to both target and non-target sites or eliminated. It has been established in several human studies that phosphorothioate ODN are highly protein-bound in the blood, largely to the major protein albumin. It is also notable that in cells grown in vitro, lipid-based carriers (liposomes) are often required for the uptake of ODN, whereas in vivo the ODN are typically effective with no carrier. What is not clear, however, is exactly what the ODN are binding in the bloodstream (other than albumin). The nature of the interaction of ODN with blood components and the factors mediating cellular uptake in tissues outside of the bloodstream is also of interest. The interaction of ODN with such components may be helpful for their activity in vivo, such as by promoting cellular uptake, which would be contrary to the usual idea of only free drug being pharmacologically active/available. It is also possible, however, that these interactions limit their usefulness by reducing the effective concentration at the target, by inducing toxicities (such as interfering with the coagulation system or complement) or by directing the ODN away from the desired target.

The results presented here show that G3139 is highly protein bound, as expected, with the majority of the ODN in the lipoprotein-deficient fraction of plasma, with or without liposomal association. After pre-incubation in human plasma, free G3139 and lipoplex G3139 induced modest complement protein (C3a) activation (Fig. 2). The ODN concentrations used herein are within the range of human plasma concentrations achieved in G3139 clinical trials (0–2 μg/ml). Free ODN has an overall negative charge, while the lipoplexes had an overall positive charge. Phosphorothioates oligonucleotides are well known to

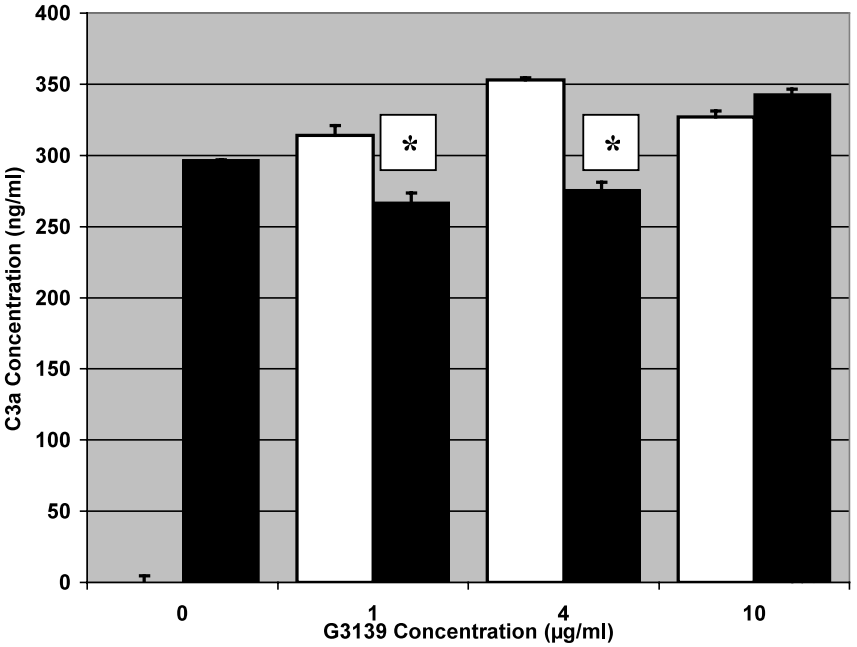

Fig. 2. Activation of human complement (C3a) by G3139 ODN and by G3139 cationic lipoplexes. Free G3139 activates C3a complement protein more than G3139 lipoplexes in human serum. White bars: Free oligodeoxynucleotides; black bars: cationic lipoplexes. Values represent mean ± S.D. ($n = 3$). *$P < 0.05$ vs. free oligodeoxynucleotides.

induce a complement response Henry et al., 1997. This has been noted in some clinical trials in a dose-response manner Rudin et al., 2001, but not others Nemunaitis et al., 1999, implying a role for the specific molecule's structure.

Pre-incubation of G3139 and G3139-lipoplexes in human plasma or serum prior to addition to tissue culture medium increased the relative association of G3139 and G3139-lipoplexes with human melanoma cells (Fig. 1). Serum appeared to increase association of free ODN more than plasma. Coagulation factors are relatively depleted in serum, but further studies will be required to determine what specific factors are involved. Thrombin, for example, is known to bind to antisense molecules, potentially increasing coagulation time Henry et al., 1994. Most clinical trials of phosphorothioate ODNs now monitor coagulation parameters. It is presently unknown if this factor is important in the fate of ODN molecules in the bloodstream. It is clear, however, that G3139 lipoplexes do exhibit altered lipoprotein binding, complement activation and cellular association compared with free G3139 and that human plasma vs. serum may have a differential effect on cellular association. In vivo, specific plasma protein binding attributes may affect access to the target tissues, a prerequisite for activity.

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