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Interaction of Oligonucleotide-Conjugates With the Dipeptide Transporter System in Caco-2 Cells

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ABSTRACT. Oligonucleotide-based therapies represent novel strategies for manipulating the expression and function of target proteins and are undergoing clinical evaluation for the treatment of viral diseases and malignancies. However, poor biological stability and cellular delivery represent potential limitations to the therapeutic development of oligonucleotides. Conjugation of oligonucleotides to lipophilic groups can improve delivery to cells but the enhanced cellular binding may also facilitate nonspecific interactions. In this report, we show that phosphorothioate oligonucleotides conjugated to lipophilic groups, either tocopherol (Vitamin E) or 2-Di-O-hexadecyl-3-glycerol, can significantly inhibit the functioning of the dipeptide transporter system (DTS) in cultured Caco-2 intestinal cells. Because the DTS mediates the binding and absorption of nutrient peptides and important drugs, such as the cephalosporin and penicillin antibiotics, this finding has important implications in relation to the potential toxicity of lipophilic conjugates *in vivo*. It also suggests a potential drug interaction with lipophilic oligonucleotide-conjugates if they were to be delivered orally. BIOCHEM PHARMACOL 53;9:1223–1228, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. oligonucleotides; antisense; phosphorothioates; dipeptide transporter system; Caco-2 cells; oral delivery

Antisense oligonucleotides have emerged as novel therapeutic agents for the sequence-specific inhibition of gene expression [1], and modified phosphorothioate oligonucleotides are currently undergoing clinical evaluation for the treatment of viral infections in AIDS patients and both chronic and acute myelogenous leukaemias [2]. However, efficient delivery of oligonucleotides to target tissues remains a problem and a number of delivery strategies and routes of administration [3], including the oral route [4], have been examined. Indeed a recent report claims up to 25% bioavailability of modified oligonucleotides when administered as a 50 mg/kg single dose to rats via the oral route [4].

In an attempt to further understand the underlying mechanisms for oligonucleotide absorption from the oral route, we have previously been studying the interactions of oligonucleotides with the gastrointestinal epithelium using cultured human intestinal (Caco-2) cells [5, 6]. Caco-2 cells express the cell-surface dipeptide transporter system (DTS) [7, 8] and have also been extensively used as a model to study the oral absorption of several dipeptide-like drugs (e.g. angiotensin-converting enzyme (ACE) inhibitors and aminocephalosporins), which utilise this cell transport system [7, 9, 10].

In the present study, we show that the uptake of a model dipeptide, glycyl-3, 4-[^{3}H]L-proline ([^{3}H]Gly-L-Pro), by the DTS in Caco-2 cells was significantly impaired in the presence of phosphorothioate oligonucleotides that have been conjugated with the delivery enhancing lipophilic moieties Vitamin E (VitE-S-rev) or 2-D-O-hexadecyl-3-glycerol (DHDG-S-rev) (for structures see Fig. 1). This effect was not observed by oligonucleotides alone or the lipophilic group alone, and suggests that lipophile-oligonucleotide conjugates could compromise the normal functioning of the DTS in mediating absorption of nutrient peptides and of some important drugs should they be considered for oral delivery.

MATERIALS AND METHODS

Cell Culture

The Caco-2 cell line (purchased from ATCC, USA) was used between passages 30 and 40. Cells were grown in 150 cm^2 plastic tissue-culture flasks, and maintained in M1 (Dulbecco's modified Eagle's medium (Gibco-BRL, Life Technologies, UK) supplemented with 4.5 g L^{-1} glucose,

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Abbreviations: ACE, angiotensin-converting enzyme; Ap, apical; DHDG, 2-D-O-hexadecyl-3-glycerol; DTS, dipeptide transporter system; PBS, phosphate-buffered saline; VitE, vitamin E; [^{3}H]Gly-L-Pro, glycyl-3, 4-[^{3}H]L-proline; SQ-29852, Ceronopril.

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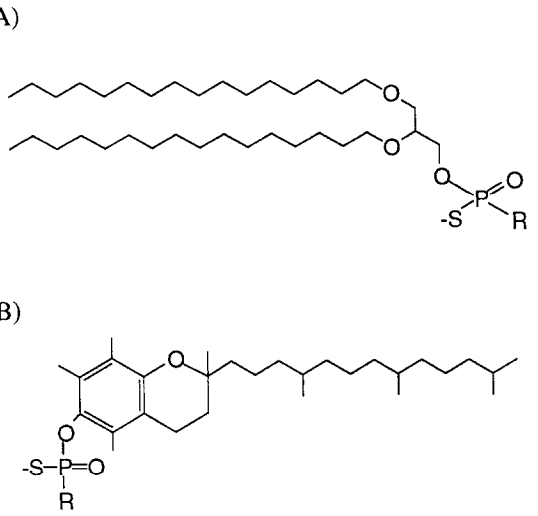

FIG. 1. Structure of lipophilic-oligonucleotide conjugates. The lipophilic moieties (A) = 2-Di-*O*-hexadecyl-3-glycerol or (B) = Vitamin E (tocopherol) were conjugated to a 28-mer-phosphorothioate oligonucleotide (S-rev) via its 5′ terminal thiophosphate where R is the oligonucleotide, 5′ TCGTCGCTGTCTCCGCTTCTTCCTGCCA 3′.

10% (v/v) foetal calf serum, 1% (w/v) nonessential amino acids, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg mL^{-1} streptomycin and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid buffer solution, pH 7.4 (Gibco-BRL, Life Technologies, UK)), which was replaced every 48 hr. The cultures were grown at 37° in an atmosphere of 5% CO_2 (95% air) and 90% relative humidity. The cultures were passaged every 3–4 days using a solution containing 0.25% (w/v) trypsin and 0.25% (w/v) ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS), pH 7.2 (Oxoid, UK) and flasks were seeded at approximately 4.7×10^4 cells cm^{-2}. For uptake studies the cells were used between passages 30 and 40 and seeded on 24-well tissue culture plates (2 cm^2) at a density of 8×10^4 cells cm^{-2} (1.6×10^5 cells $well^{-1}$). The M1 (2 ml) was renewed every 48 hr and the monolayers were used after 7 days of growth. The incubation medium (M2) comprised Hank's balanced salt solution (Gibco-BRL, Life Technologies, UK) containing 25 mM 2-[*N*-morpholino]ethanesulphonic acid adjusted to pH 6 using NaOH (1 M), unless otherwise stated.

Oligodeoxynucleotide Synthesis

The 28mer phosphorothioate oligodeoxynucleotide (S-rev; 5′ TCG TCG CTG TCT CCG CTT CTT CCT GCC A 3′) complementary to the *rev* gene in HIV-1 was synthesized on an ABI 392 automated DNA synthesizer (Applied BioSystems, UK) using standard phosphoramidite chemistry, with tetraethyl thiuram disulphide used as the sulphurizing reagent. VitE-S-rev and DHDG-S-rev conjugates were synthesized as described previously [11, 12] and purified by HPLC on a Brownlee Aquapore small rp 300 C8 (octyl) reversed-phase column using a gradient of 0–60% (v/v) acetonitrile in a buffer of 0.1 M ammonium acetate (pH 7.0). After HPLC, solvent was removed on a rotary evaporator and the oligonucleotide was dissolved in 1 mL of water and freeze dried. It was then desalted by sephadex G25 gel filtration. The oligonucleotide was 5′ end [^{32}P]-labelled with T4 polynucleotide kinase and purified as described previously [5, 6, 13].

Interactions With [^{3}H]Gly-L-Pro Uptake via the DTS Into Caco-2 Cells

The presence of a functional DTS was confirmed in Caco-2 cells by studying the uptake of the well-characterized substrate, a dipeptide probe glycyl-3, 4-[^{3}H]L-proline ([^{3}H]Gly-L-Pro) (50 Ci $mmol^{-1}$) and competition with SQ-29852 (Ceronopril; an ACE inhibitor) (both were gifts from Bristol-Myers Squibb, USA). A rationale and full description of the methods used and validation of the system have been described previously [10]. Briefly, the experimental conditions used for the competition studies are as follows; in all experiments M2 contained 62.5 nM of the probe [^{3}H]Gly-L-Pro and 10 mM L-proline at pH 6 (unless otherwise stated), with the required amount of oligonucleotide conjugate. On each experimental day, SQ-29852 was tested at 1 mM concentration to check the reproducibility and viability of the system. All solutions used were preheated at 37°, with the exception of PBS/0.05% (w/v) sodium azide, which was kept at 4°. Cells were washed (500 μL for 10 min) with M2 at 37° and the incubation solution added. Two different incubation procedures were used: (1) incubation with 200 μL incubation solution for 3 min, and (2) preincubation with 200 μL of incubation solution for 15 min, and then addition of the probe to each well and incubation for a further 3 min. At the end of the incubation period, the plates were transferred to a cold room (4°) and the apical (Ap) solutions were removed. The monolayers were washed (2 × 500 μL × 5 min) with PBS/0.05% (w/v) sodium azide (4°), and then harvested with 1 mL of 1% (v/v) Triton X-100 in double distilled water (10 min, 37°). The radioactivity associated with the Ap solutions (200 μL, made up to 1 mL with double distilled water), washings (1 mL) and solubilised cells (1 mL) was quantified by liquid scintillation counting (10 mL Hisafe 3 scintillation cocktail, 10 min) (LSC 2500 counter, Hewlett Packard, UK).

Data Analysis

The data sets are expressed as the mean ± SD from at least three monolayers. Results are expressed as uptake of [^{3}H]Gly-L-Pro (pmol mg $protein^{-1}$), or % inhibition of the control. In the figures,* denotes significant inhibition from the control value. Statistical analysis were carried out using an unpaired *t*-test with 95% confidence limits.

RESULTS AND DISCUSSION

The cellular uptake mechanisms and intracellular fate of oligonucleotides have recently been reviewed by Akhtar

and Juliano [14]. The generally inefficient uptake of unconjugated oligonucleotides by endocytosis has led to the investigation of alternative delivery strategies for oligonucleotides, such as the use of lipophilic conjugates (for reviews see [3]). It has previously been shown that lipophilic 5′ cholesterol modification to phosphorothioate oligonucleotides increases the intracellular accumulation by direct insertion of the cholesteryl moiety into the cell membrane [15], and by binding to low-density lipoprotein (LDL) with subsequent internalisation via the LDL receptor [15, 16]. However, the enhanced cellular binding of lipophilic oligonucleotide conjugates can also lead to nonspecific interactions with cell-surface proteins such as the drug transporters (e.g. DTS). The aim of this study was to investigate the likely nonspecific interactions of lipophilic VitE-S-rev and DHDG-S-rev conjugates in influencing the efficiency of uptake of nutrients or drugs via the DTS in Caco-2 cells. In the present work, the DTS expressed by Caco-2 cells was simply used as a model process. The DTS, in addition to mediating the uptake of di- and tri-peptide fragments of digested nutrient proteins, plays an essential role in mediating the absorption of important dipeptide-like drugs such as ACE inhibitors, renin inhibitors, cephalosporins, and several penicillin antibiotics [7, 9]. The DTS is thought to be expressed in most regions along the gastrointestinal tract including the duodenum, jejunum, ileum, and colon [8]. The acidic nature of the gastrointestinal tract provides the proton gradient, which is the driving force for the DTS, a protein of about 708 amino acids consisting of 12 *trans*-membrane spanning domains [17]. Although the luminal pH is around pH 6, there is considerable evidence that suggests that an acid microclimate exits at the surface of the gastrointestinal tract and where pHs as low as pH 5 or pH 5.5 have been reported in both rats and humans [18–21]. Indeed, the existence of this acid microclimate and alterations in its pH is thought to explain why the weakly acidic drug folic acid undergoes malabsorption in Coeliac disease [22]. As a result of this reported pH range within the gastrointestinal tract, we studied the interaction of oligonucleotide conjugates at both pH 5 and pH 6.

Figure 2 shows the functional presence of the DTS in mediating uptake of the dipeptide, [^{3}H]Gly-L-Pro, at pH 6 in Caco-2 cells. This dipeptide substrate was chosen as a probe because it is relatively stable to enzymatic digestion and displays high affinity for the transporter (K_m of 389 ± 15 nM) [10]. We have previously shown that probe degradation is less than 7% during the short incubation times (3 min) used in this study [10]. Nonetheless, as a precautionary measure, to prevent erroneous uptake readings through hydrolysis of the dipeptide to [^{3}H]L-proline monomer and its subsequent internalization via the saturable imino acid transporter into Caco-2 cells [23], an excess concentration of L-proline (10 mM) was included in the uptake studies as described previously [10]. The control uptake of the [^{3}H]Gly-L-Pro probe in Caco-2 cells was 1.3 ± 0.1 pmol/10^6 cells and values are normalised to this value (100%) in Fig.

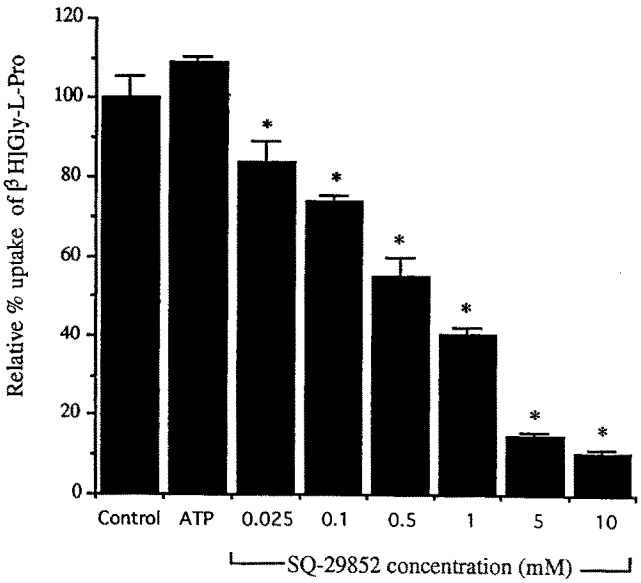

FIG. 2. The effect of increasing concentrations of SQ-29852 on the uptake of [^{3}H]Gly-L-Pro into Caco-2 cell monolayers. Uptake of [^{3}H]Gly-L-Pro in the presence of 10 mM L-proline (Control), 1 mM ATP and in the presence of increasing concentrations of SQ-29852; (0.025–10 mM) is shown. The results (mean from at least three monolayers ± SD) are expressed as % inhibition of the control (62.5 nM [^{3}H]Gly-L-Pro and 10 mM L-proline). *Donates significant inhibition at 95% confidence limits compared to control value.

2. To confirm that uptake of this dipeptide was mediated by the DTS, a dose-dependent inhibition, with an IC_{50} of 676 ± 6 mM, which is similar to that reported previously [10], was achieved using the ACE inhibitor, ceronopril (SQ-29852), a known inhibitor of the DTS [24]. The functioning of the DTS was unaffected by the presence of 1 mM ATP (Fig. 2) or by competition with 1 mM concentrations of L-lysine, L-serine, or taurocholic acid (data not shown) confirming that mononucleotides, amino acids, and bile acids are not substrates of the DTS. This finding also indicates that the uptake of the labelled probe is not being mediated by the amino acid or bile acid transport systems [25, 26].

Figures 3 and 4 show that, depending on the pH, up to 80% of the DTS function in mediating uptake of the [^{3}H]Gly-L-Pro substrate could be inhibited in the presence of lipophilic oligonucleotide-conjugates. At pH 6 (Fig. 3) the simultaneous addition of probe with oligonucleotides (black bars) or the addition of probe following a 15-min preincubation of oligonucleotide-conjugates (white bars) caused similar effects. The 15-min preincubation time was chosen as we have previously demonstrated that oligonucleotide binding to Caco-2 cells is complete within this time period [5]. A dose-dependent inhibition with VitE-S-rev (up to approx 40% at 25 μM) was observed, but only a modest inhibition of the DTS with the DHDG-S-rev was noted at this pH (Fig. 3). Little or no inhibition with the unconjugated S-rev oligonucleotide or tocopherol (Vitamin E) alone was observed suggesting that the inhibitory

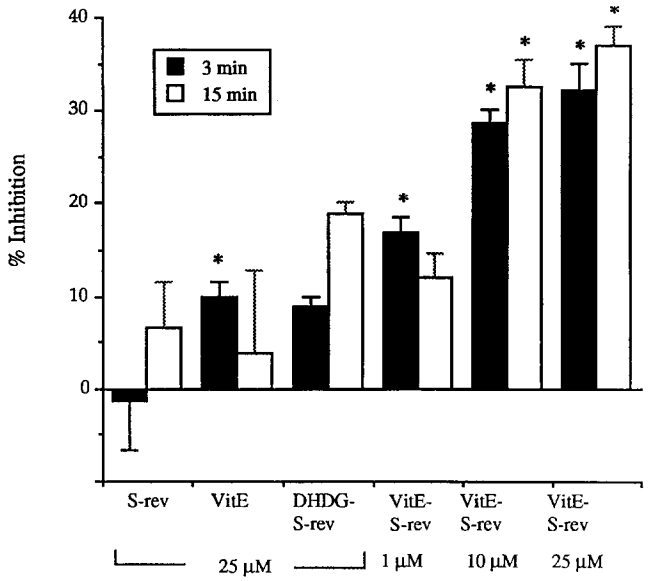

FIG. 3. The effect of competitors on the uptake of [^{3}H]Gly-L-Pro into Caco-2 cell monolayers at pH 6. Uptake of [^{3}H]Gly-L-Pro was monitored in the presence of one of the following compounds; 25 μM S-rev, 25 μM VitE, 25 μM DHDG-S-rev, 1 μM, 10 μM, and 25 μM VitE-S-rev over a 3-min period when the competitors were added simultaneously with the probe (black bars) or when competitors were added 15 min prior (white bars). The results (mean from at least three monolayers ± SD) are expressed as % inhibition of the control (62.5 nM [^{3}H]Gly-L-Pro and 10 mM L-proline). *Donates significant inhibition at 95% confidence limits from appropriate control values ([^{3}H]Gly-L-Pro uptake after 3 min with or without a 15 min preincubation stage).

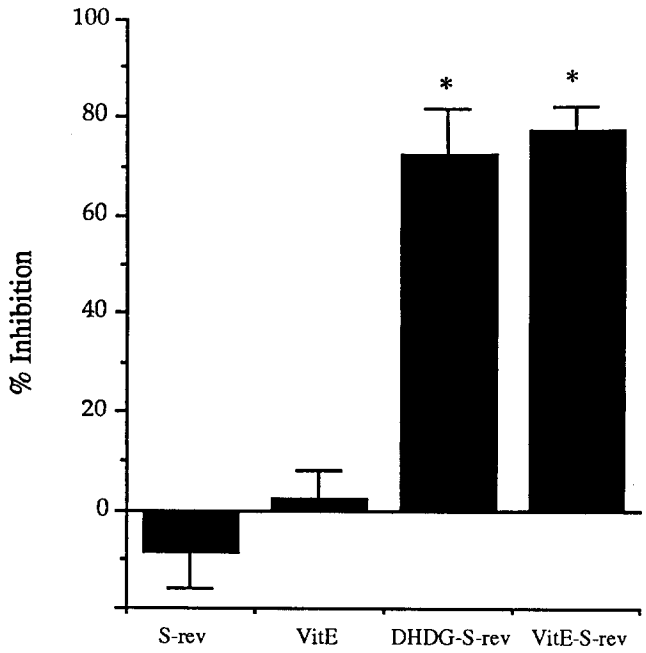

FIG. 4. Inhibition of DTS function by oligonucleotide conjugates at pH 5. The competitors at 25 μM concentration used were S-rev, VitE, DHDG-S-rev, VitE-S-rev. Experimental conditions were as described in Fig. 3 and Materials and Methods. *Denotes significant inhibition at 95% confidence limits from control value ([^{3}H]Gly-L-Pro uptake).

effect required the oligonucleotide-VitE lipophilic conjugate (Fig. 3). Inhibition of the DTS was much more pronounced at pH 5 (Fig. 4) with both VitE-S-rev and DHDG-S-rev conjugates showing similar and enhanced activity.

Because phosphorothioate oligonucleotides bind more avidly to Caco-2 cells with decreasing pH (pH 6–5), the enhanced cell surface binding of oligonucleotide conjugates may explain the dramatically increased inhibition of the DTS at pH 5. Although the predicted Hansch values (Log P of approximately 9.6 and 11.1, for VitE and DHDG moieties, respectively) [27] indicate that these molecules are highly lipophilic, their ionic character is unlikely to change over this pH range. Therefore, it is possible that the enhanced affinity of the conjugates for the DTS at pH 5 compared to pH 6, is due to the protonation of histidine residues present in the DTS, as has been suggested for other oligonucleotide binding proteins [5].

In an experiment designed to investigate whether VitE-S-rev conjugate bound to the same sites as the ACE inhibitor, we coadministered ceronopril (SQ-29852) with the VitE-S-rev conjugate in our DTS assay system. Coadministration of 1 mM SQ-29852 and 25 μM VitE-S-rev produced 49.54 ± 6.36% inhibition of Gly-[^{3}H]L-Pro uptake (Fig. 5). The inhibition observed was not significantly different (P = 0.01) from that seen when administering SQ-29852 alone (57.13 ± 3.09%). Similar results were observed when SQ-29852 was preadministered 15 min before the conjugate (data not shown). As there clearly was no additive effect, these results suggested that VitE-S-rev inhibited

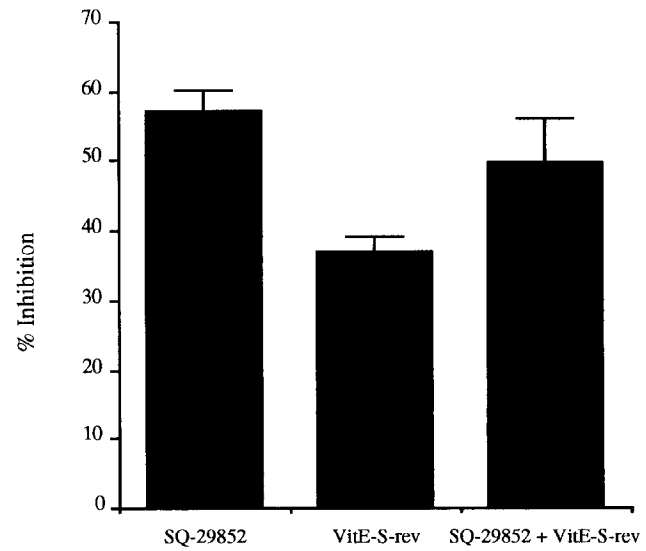

FIG. 5. The effect of coadministering SQ-29852 and VitE-S-rev on the uptake of Gly-[^{3}H]L-Pro via the DTS into Caco-2 cell monolayers. Uptake of the dipeptide probe was monitored at pH 6 in the presence of either 1 mM SQ-29852 alone, 25 μM VitE-S-rev alone, or when coadministered together. Data are expressed as mean (of at least three monolayers) ± SD.

DTS function by binding to the same sites (on the DTS) as SQ-29852, or possibly that SQ-29852 has a greater affinity for these sites than the lipophilic oligonucleotide conjugate.

In the above studies, concentrations of oligonucleotides and their conjugates of up to 25 μM were examined for their effects on DTS function. Although the extent to which the conjugates are absorbed orally is not known, the reported oral bioavailability of oligonucleotides is only between 12–25% [4]. The chosen concentration range, therefore, reflects the relatively high doses that will be necessary to achieve clinically therapeutic concentrations (in the nano-to-micromolar range) of these agents within individual tissues and cells via the oral route. Thus, the gastrointestinal tract is likely to encounter much higher concentrations of these agents, at least initially, than tissues elsewhere in the body should oligonucleotides and/or their conjugates be considered for oral administration.

The above results taken together suggest that the extent and nature of the unconjugated S-rev binding to Caco-2 cells is insufficient to cause inhibition of the DTS. We have previously shown that, in Caco-2 cells, phosphorothioate oligonucleotides bind predominantly to a 46 kDa cell-surface protein, although some binding was also associated with a 110–120 kDa protein [6]. Although the identity of this latter protein is not known, its molecular weight approximates to that of the DTS [8]. Thus, the inhibitory effects of the lipophilic conjugates may represent an enhanced affinity of the conjugated oligonucleotide for this protein. The exact binding sites for the conjugates are not known but may involve both proteins [6] and, because some oligonucleotide derivatives interact with lipid vesicles [13], lipid binding sites as well. Thus, at pH 6 it appears that VitE-S-rev must have a greater affinity for binding to the DTS (for competitive inhibition effect) or possibly to surface proteins and/or lipids regulating the functioning of the DTS (allosteric inhibition) than DHDG-S-rev, whereas at pH 5 both conjugates exhibited an equal capacity to interfere with the DTS, indicating an enhanced affinity for cell surface sites regulating DTS function.

The greater improvement in inhibitory effect noted for the DHDG-S-rev conjugate over this pH range may be due to changes in the ionic environment of either the binding proteins/lipids at the cell surface or to the conjugate itself. This is consistent with the enhanced binding of modified oligonucleotides to Caco-2 cells at low pH, which is mediated in part by an altered spectrum of oligonucleotide binding proteins at pH 5 [6]. However, the exact nature and mechanism of these interactions clearly require further study.

In conclusion, we have demonstrated that lipophilic oligonucleotide-conjugates designed to improve cellular delivery can also exert nonspecific and potentially adverse effects. In this case, we have shown that VitE-S-rev and DHDG-S-rev, especially at the lower pH, can dramatically affect the functioning of the DTS in Caco-2 cells. This effect could potentially compromise the absorption of nutrient peptides and important drugs should oligonucleotide-conjugates be administered orally [4], and/or possibly interfere with absorption profiles in nontarget cells upon systemic administration.

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