

# Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3'-terminus and their interaction with DNA within mammalian cells

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5'-[<sup>32</sup>P]-labelled alkylating decathymidylate [4-(*N*-2-chloroethyl)*N*-methylaminobenzyl]-5'-phosphamide derivatives containing cholesterol or phenazinium residues at their 3'-termini were synthesized and used for alkylation of DNA within mammalian cells. The uptake of the cholesterol derivative by the cells and the extent of DNA alkylation are about two orders of magnitude higher than those of a similar alkylating derivative lacking the groups at the 3'-termini. The presence of the phenazinium residue at the 3'-terminus of the oligonucleotide reagent does not improve the reagent uptake by the cells but drastically increases the DNA modification efficiency.

Reactive oligonucleotide derivative; Oligonucleotide uptake; DNA affinity modification; Hydrophobic group; Polyaromatic group

## 1. INTRODUCTION

Oligonucleotide derivatives are considered as potential biologically active compounds capable of interfering with functions of target nucleic acids possessing complementary nucleotide sequences [1]. Antisense oligonucleotide derivatives can be used to arrest translation of mRNA or to suppress viruses multiplication [1–7]. Recent data on the interaction of oligonucleotide derivatives with superhelical plasmid DNA, DNA in isolated chromatin and within living cells [7–11] suggest the principal possibility of sequence-specific chemical modification of genomic DNA. The efficiency of oligonucleotide derivatives as reagents targeted to cellular nucleic acids can be increased by improv-

ing their ability to penetrate into cells and by stabilizing their complexes with the target nucleic acids.

In the present paper, we report the synthesis of alkylating oligonucleotide derivatives bearing cholesterol groups facilitating the binding of the derivatives to the cellular membrane, and phenazinium groups stabilizing the complexes of oligonucleotides with complementary nucleic acids. It was found that these derivatives react with cellular DNA much better than similar alkylating derivatives lacking polyaromatic or hydrophobic groups.

## 2. MATERIALS AND METHODS

Chemicals and enzymes were from Sigma and Merck. CIRCH<sub>2</sub>NH<sub>2</sub> was obtained from Novosibirsk Institute of Organic Chemistry, Siberian Division of the USSR Academy of Sciences. [ $\gamma$ -<sup>32</sup>P]ATP of a high specific activity (>6000 Ci/mmol) was obtained from the Institute of Nuclear Physics, Uzbek SSR Academy of Sciences. Decathymidylate was synthesized by the phosphotriester method in solution [12,13]. Protected oligonucleotide was phosphorylated [14] and cholesterol

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*Abbreviations:* ATP, adenosine 5'-triphosphate; CIRCH<sub>2</sub>NH<sub>2</sub>, [4-(*N*-2-chloroethyl)*N*-methylaminobenzyl]-amine

or amino-protected aminoethanol was coupled to the 3'-phosphate of the oligonucleotide to yield corresponding 3'-phosphodiester. The oligonucleotide derivatives synthesized were unblocked and isolated. Phenazinium was coupled to decathymidylate, carrying an amino group at the 3'-end, under conditions described by Zarytova et al. [15]. The oligonucleotide derivatives were labelled by T4 polynucleotide kinase-catalyzed phosphorylation with 5'-[ $\gamma$ - $^{32}$ P]ATP [16] and ClRCH<sub>2</sub>NH<sub>2</sub> was coupled to the 5'-phosphate of the derivative according to Godovikova et al. [17]. Details of the synthetic and purification procedures will be published elsewhere.

Cultivation of L-929 mice fibroblasts and Krebs-2 ascite carcinoma cells was described earlier [11,18]. After treatment of the cells with the oligonucleotide derivatives, the cell lysates were analyzed by gel electrophoresis in agarose or 5–20% polyacrylamide gel followed by ethidium bromide staining and radioautography. Experiments with chromatin were carried out on the isolated rat liver nuclei [19]. DNA was isolated by phenol extraction, treated by proteinase K and ribonuclease A, and analyzed by gelfiltration on a Sephadex G-100 column in 7 M urea or by electrophoresis in 1% agarose and radioactivity bound with DNA was counted in a liquid scintillation system.

### 3. RESULTS AND DISCUSSION

Recent studies [1–6,11,18] have demonstrated that deoxyribonucleotides can penetrate eukaryotic cells by the adsorption and liquid endocytosis mechanism [18]. In order to investigate

the possibility of improving oligonucleotide uptake by coupling the hydrophobic groups, we synthesized reagents I, II, and III (fig.1) and investigated their interaction with Krebs-2 ascite carcinoma cells and L-929 mouse fibroblasts. The results of the experiments are represented in fig.2. The most efficient binding was observed for the cholesterol derivative at 37°C.

Fig.3 shows the dependence of the steady-state

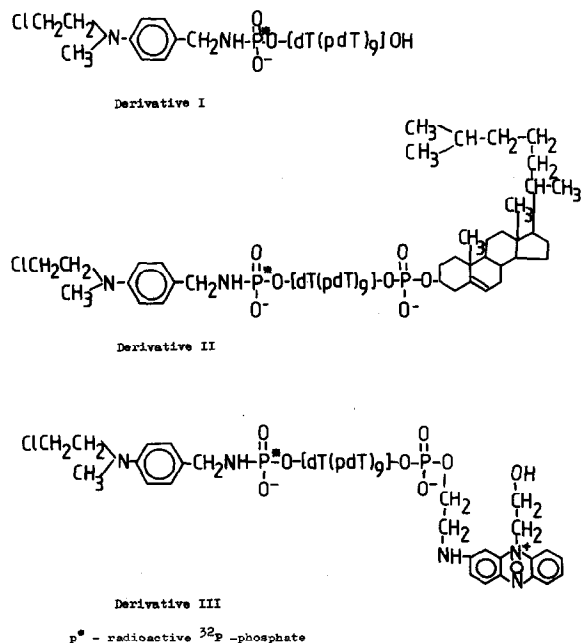


Fig.1. Alkylating oligonucleotide derivatives.

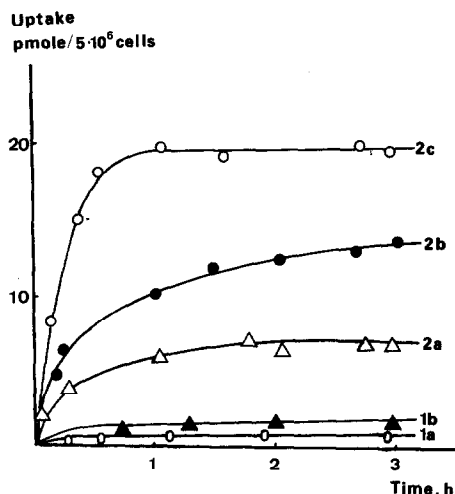


Fig.2. Kinetics of the reagents I (1) and II (2) uptake by the Krebs-2 ascite carcinoma cells in RPMI medium at different temperatures: (a) 5°C; (b) 20°C; (c) 37°C. Cell concentration,  $5 \times 10^6$  per ml; reagent concentrations, 0.5  $\mu$ M.

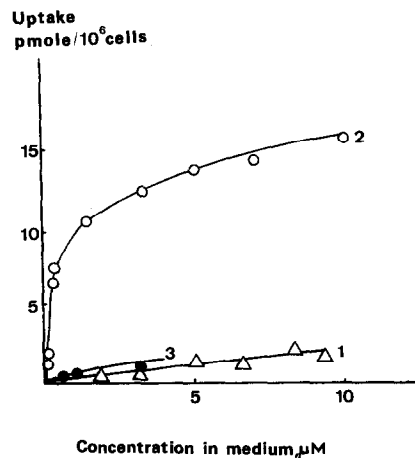


Fig.3. Effect of oligonucleotide derivative concentrations on their limit of uptake (incubation for 2 h at 37°C): 1, reagent I; 2, reagent II; 3, reagent III.

Table 1  
Oligonucleotide derivative uptake by Krebs-2 ascite carcinoma cells and L-929 mice fibroblasts.

Cells	Reagent	Concentration ( $\mu\text{M}$ )		Uptake by the cells (pmol per $5 \times 10^6$ cells)	Contents in the nuclei (pmol per $5 \times 10^6$ cells)
		In the medium	In the cells <sup>a</sup>		
Krebs 2 ascite carcinoma	I	0.5	0.16	0.9	0.057
	II	0.5	4.0	23.5	7.68
	III	0.5	0.13	0.7	0.11
L-929 mice fibroblasts	I	1.0	1.0	6.0	0.27
	II	2.5	15.0	90.0	3.6
	III	2.0	2.3	13.8	0.62

<sup>a</sup>The cell volume was assumed to be  $1.1\text{--}1.2 \times 10^{-6} \mu\text{l}$  [11]. The accuracy of concentration determination was about  $\pm 15\%$ . The cells were incubated with the derivatives in RPMI medium for 2 h at  $37^\circ\text{C}$ .

intracellular oligonucleotide concentration (after reaching the plateau) on the concentration of the derivative in the medium. It is seen that the cholesterol residue causes a considerable increase of the oligonucleotide uptake level. Phenazinium residue has no effect on the derivative uptake. In table 1, intracellular concentrations and the level of the oligonucleotide derivative uptake are shown. It is seen that all the derivatives penetrate the cells and reach the nucleus, the highest uptake level being observed for reagent II. Its concentration within the cell is about 8 times higher than in the medium, and substantial amounts of the derivative are found in the nucleus. It is possible that some cholesterol-binding proteins [20] can take part in the delivery of cholesterol-containing reagent into the nucleus. From this point of view, interesting results were obtained in the experiments with chromatin in the isolated mice nuclei (table 2). The modification level of the chromatin DNA treated by reagent II was found to be one order of magnitude higher than that of DNA treated by reagent I under similar conditions.

Table 2

Extent of modification of DNA in chromatin of the isolated rat liver nuclei (mol of reagent per mol of nucleotides  $\times 10^6$ )

Reagent	Concentration of competitor (pT) <sub>10</sub> ( $\mu\text{M}$ )	Extent of modification
I	–	$18 \pm 4$
	4	$4 \pm 8$
II	–	$186 \pm 20$
	17	$50 \pm 9$

Nuclei ( $10^7$  per ml) were treated with the reagents ( $1 \mu\text{M}$ ) in a buffer for nuclei isolation [19]

Electrophoresis data (not shown) indicated that up to 70% radioactivity was bound with the biopolymers. The radioactive material was found in DNA. Treatment of DNA with an  $\text{HClO}_4$  solution resulted in the release of DNA-bound radioactivity thus indicating that it was associated with DNA due to chemical modification and not due to reutilization of radioactive phosphate. The DNA modification level was determined as a specific DNA-bound radioactivity after it was additionally treated by protease, RNase and purified by gel filtration under denaturing conditions. When the cells were treated with hydrolyzed reagent analogue (i.e. containing OH group instead of Cl), no radioactivity was eluted with the cellular DNA. The extent of modification appeared to be 0.79 mol per  $10^6$  mol of nucleotides for reagent I, 54 mol for reagent II, and 6.5 mol for reagent III.

The increase in DNA modification efficiency for reagents II and III has probably been brought about differently. In the case of reagent II, two factors may play an important role: efficient binding of the hydrophobic reagent with the cellular membrane and a higher affinity of cholesterol to chromatin, probably mediated by some cholesterol-binding proteins. The polyaromatic residue in reagent III stabilizes the complementary complex formed by the derivative. Therefore, the modification level achieved with the reagent is quite high despite the low uptake efficiency of this derivative.

The results obtained provide evidence that the coupling of lipophilic membrane-anchoring groups to oligonucleotides facilitates the delivery of oligonucleotide derivatives into mammalian cells.

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