

Novel Nicotinic Acetylcholine Receptor Inhibitors Derived from Oleoylcholine Analogs

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ABSTRACT Fatty acid-acylated cholines, a recently identified class of endogenous compounds, have been detected in both human and animal organisms. Our prior work established that oleoylcholine (Ol-Chol), and other acylcholines, at micromolar levels, modulate the cholinergic system and are suitable as cationic lipids for introducing nucleic acids into human and animal cells. The present research examines the interaction with the nicotinic acetylcholine receptors (nAChR) of two ionic forms of Ol-Chol and two synthesized cationic lipids, each featuring a quaternary ammonium moiety and two oleic acid chains. A radioligand binding assay revealed that the affinity of acylcholines and synthetic cationic lipids for the muscle-type nAChR surpasses that for the human neuronal $\alpha 7$ nAChR by a factor of 2–5.5. Oleoylcholine iodide demonstrated a two-fold higher efficacy of mesylate in binding to the orthosteric site of muscle and $\alpha 7$ nAChR. In a functional calcium imaging assay, both compounds exhibited superior inhibition of $\alpha 7$ nAChR by several orders of magnitude, suggesting potential interaction with allosteric binding sites. Compared to oleoylcholine, synthetic cationic lipids demonstrated markedly reduced efficacy in binding to $\alpha 7$ nAChRs and, in contrast to oleoylcholine, induced a substantial cytotoxic impact on SH-SY5Y neuroblastoma cells, a phenomenon unaffected by specific nAChR ligands. As a result, the nAChR-inhibitory properties are attributed to the quaternary ammonium group present in all studied compounds. However, the modification of the lipophilic moiety with two oleic acid residues curbs these properties but enhances cytotoxic activity through an alternative mechanism independent of nAChR.

KEYWORDS acylated cholines, nicotinic acetylcholine receptor, inhibitor, DOTAP, SH-SY5Y, cytotoxicity.

ABBREVIATIONS nAChR – nicotinic acetylcholine receptor; α Bgt – α -bungarotoxin from the venom of *Bungarus multicinctus*; [¹²⁵I]- α Bgt – α -bungarotoxin, labeled with the iodine-125 isotope; CTX – α -cobratoxin from *Naja kaouthia* venom; LDH – lactate dehydrogenase; Ol-Chol⁺I⁻ – oleoylcholine iodide; Ol-Chol⁺Mes⁻ – oleoylcholine mesylate; DOTAP⁺Mes⁻ – (1,2-Bis-(9Z-octadecenoyloxy)-3-trimethylammonium propane mesylate; DOGG-Chol⁺I⁻ – choline ester iodide of 4-((((1,3-bis(oleoyloxy)propan-2-yl)oxy)carbonyl)amino)butanoic acid; Nic – nicotine; Mec – mecamlamine; dTC – d-tubocurarine; MLA – methyllycaconitine; GABA – gamma-aminobutyric acid.

INTRODUCTION

Acetylcholine analogs, defined as choline esters of unsaturated fatty acids, have been detected in human plasma and urine [1]. Furthermore, elevated levels of long-chain unsaturated acylcholines are observed in specific pathological conditions. For instance, choline esters of oleic, linoleic, and arachidonic acids have been detected within the vascular tissues of individuals with cardiovascular conditions, specifically abdom-

inal aortic aneurysms, atherosclerotic plaques in the carotid arteries, atherosclerotic plaques in the femoral arteries, and intimal thickening [2, 3]. Acylcholines, specifically their unsaturated forms, are present in elevated concentrations in the blood of individuals at a heightened risk for pulmonary embolism (PE) when contrasted with those at a moderate risk for the condition [4]. Accordingly, it is plausible that excessive accumulation of acylcholines is linked to the etiology

of a range of human diseases. However, the precise mechanism by which acylcholines operate is not yet well-established.

Our prior research indicated that specific endogenous acylcholines, including arachidonoylcholine, oleoylcholine (Ol-Chol), linolenoylcholine, and docosa-hexaenoylcholine, exhibit inhibitory effects on muscle and $\alpha 7$ neuronal nicotinic acetylcholine receptors (nAChRs), as well as on acetylcholine-hydrolyzing enzymes, at micromolar concentrations [5]. This finding substantiates the hypothesis that the biological action of endogenous acylcholines is associated with their regulatory influence on acetylcholine-mediated signaling pathways. Endogenous acylcholines may potentially function as inhibitors of the oncogenic process in cancer cells that exhibit sensitivity to $\alpha 7$ -nAChR inhibition, such as in lung cancer [6].

Due to their cationic lipid nature and the presence of a quaternary ammonium group, acylcholines can be used to deliver nucleic acids into mammalian cells [7]. DOTAP (1,2-bis-(9Z-octadecenyl)oxy)-3-trimethylammoniumpropane) is a cationic lipid that has gained widespread popularity in the field of transfection research due to its ability to facilitate the transfer of genetic material into cells, as evidenced by numerous studies [8, 9]. This compound contains a charged quaternary ammonium group, a structural element that is analogous to choline esters. Despite the structural parallels between DOTAP and choline esters, the potential for this cationic lipid to engage with acetylcholine receptors has not yet been investigated. Furthermore, our findings suggest that the efficiency of cellular transfection is dictated by the counterion present in the cationic lipid [7]. However, the impact of counterion properties on the inhibitory action of acylcholine on nAChRs remains to be elucidated.

In this study, we investigated the interaction of two ionic forms of Ol-Chol and two synthetic cationic lipids containing a quaternary ammonium group and two oleic acid residues with muscle and neuronal $\alpha 7$ nAChRs. To this end we synthesized two forms of oleoylcholine: iodide and mesylate. In addition, two other compounds were prepared: DOTAP mesylate and a new DOTAP analog containing a choline group at a certain distance from the lipophilic dioleoylglycerol residue: the choline ester iodide 4-(((1,3-bis(oleoyloxy)propan-2-yl)oxy)carbonyl)amino)butanoic acid (*Fig. 1*). In contrast to oleoylcholine, both compounds contain two unsaturated fatty (oleic) acid residues.

nAChRs are ligand-gated membrane cation channels [10]. Muscle-type nAChRs are located on muscle fibers at neuromuscular junctions and are responsible for transmitting nerve impulses to effector cells, while

$\alpha 7$ nAChRs are widely distributed throughout the body and are present on both nerve cells and other cell types, such as immune cells, glial cells, epithelial cells, and others [11–13]. $\alpha 7$ nAChRs modulate a wide range of cellular processes, including the release of neurotransmitters, cytokines, and neurotrophic factors, as well as subsequent signal transduction, gene expression, and other processes [11, 14]. Dysfunction of muscle nicotinic acetylcholine receptors has been associated with the development of myasthenia gravis. In contrast, $\alpha 7$ nicotinic acetylcholine receptors have been linked to a variety of conditions, including neurodegenerative and psychiatric disorders, chronic pain, sepsis, rheumatoid arthritis, and cancer [6, 12, 15]. Consequently, $\alpha 7$ nAChR is regarded as a molecular target for drug development and targeted drug delivery [16, 17].

EXPERIMENTAL

Materials

The following materials were used: dioleoyl glyceride (courtesy of E.L. Vodovozova, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences), N,N'-disuccinimidyl carbonate (DSC, Sigma-Aldrich, USA), gamma-aminobutyric acid (Acros Organics, Germany, > 99%), bis(trimethylsilyl)trifluoroacetamide (BSTFA, Acros Organics), Kieselgel 60 (Merck, USA), triethylamine, iodomethane (Acros Organics, > 99%), 1,1'-carbonyldiimidazole (1,1'-CDI, Fluka, Switzerland), dimethylaminopyridine (DMAP) (Fluka, Switzerland), dimethylaminoethanol (DMAE) (Sigma-Aldrich), sodium bisulfate (Fluka), anhydrous Na₂SO₄, ethyl acetate, benzene, dichloromethane, acetonitrile, acetone, HCl, NaCl (Himed, Russia).

The following reagents were used: the Fluo-4 Direct Calcium Assay Kit (ThermoFisher Scientific, USA), along with resazurin (Macklin Inc., Shanghai, China) and a variety of nAChR ligands, including nicotine (Sigma-Aldrich), mecamylamine, d-tubocurarine, d-TC, methyllycaconitine, MLA, PNU120596, and PNU282987 (Tocris, UK). Additionally, the human neuroblastoma cell line SH-SY5Y (Sigma-Aldrich) was employed. The preparation of electric organ membranes from *Torpedo californica* was provided by F. Hucho (Free University of Berlin, Germany). The GH4C1 cell suspension was supplied by Eli Lilly, UK.

Synthesis of the iodide of the choline ester of 4-(((1,3-bis(oleoyloxy)propan-2-yl)oxy)carbonyl)amino)butanoic acid (DOGG-Chol⁺I⁻)

A solution was prepared by dissolving dioleoyl glycerol (100 mg, 0.16 mmol) in 1 mL of chloroform, followed by the addition of N,N'-DSC (45 mg,

0.18 mmol) and triethylamine (48 μ L, 0.48 mmol). The mixture was stirred at 25°C for 20 minutes until the components were completely dissolved (Solution 1). Bis(trimethylsilyl)trifluoroacetamide (0.5 mL, 1.94 mmol) was added to a suspension of gamma-aminobutyric acid (50 mg, 0.5 mmol) in acetonitrile (500 μ L), followed by stirring at 25°C until complete dissolution of the gamma-aminobutyric acid. Subsequently, the resultant solution was incorporated into Solution 1. The reaction mixture was stirred for 18 h at +4°C. Extraction of the mixture was accomplished using ethyl acetate, followed by washing the extract with a 2 N sodium bisulfate solution, water, and a saturated NaCl solution. The extract was then dried with anhydrous Na₂SO₄, filtered, and concentrated through evaporation. The substance was obtained in the amount of 118 mg, presenting as a pale-yellow oil. The target product was purified via column chromatography utilizing a Kieselgel 60 silica gel, employing a benzene–ethyl acetate gradient from 0 to 15% ethyl acetate. An amount of 47 mg of 4-(((1,3-bis(oleoyloxy)propan-2-yl)oxy)carbonyl)amino)butanoic acid (DOGG) was isolated in 39% yield as a colorless oil. ¹H NMR (CDCl₃; m, J, 300 MHz) 0.90 (6H, s, 2H18), 1.28–1.32 (40H, m, 2H4–7; 2H12–17), 1.63 (4H, m, 2H3), 1.88 (2H, H3' (GABA)), 2.03 (8H, m, 2H8; 2H11), 2.34 (4H, 2H2), 2.43 (2H, H2' (GABA)), 3.27 (2H, m, H4'), 4.20–4.28 (4H, dm, 2H2' (glycerol)), 4.89 (1H, H1'' (glycerol)), 5.36 (4H, m, 2H9; 2H10).

The next reaction involved dissolving 36 mg (0.05 mmol) of DOGG in 500 μ L of methylene chloride, followed by the addition of 10 mg (0.06 mmol) of 1,1'-CDI and 8 μ L (0.06 mmol) of triethylamine. This mixture was then stirred at 22°C using a magnetic stirrer for a duration of 60 minutes. A total of 1.8 mg (0.015 mmol) of DMAP and 10 μ L (0.1 mmol) of DMAE were added to the prepared imidazolide of 4-(((1,3-bis(oleoyloxy)propan-2-yl)oxy)carbonyl)amino)butanoic acid, and the resultant mixture was stirred for 18 h at 22°C under an argon atmosphere. Following dilution of the reaction mixture with chloroform, it underwent sequential washes with 0.1 N HCl and H₂O. The organic phase was then treated with a saturated NaCl solution and dried using an anhydrous Na₂SO₄. The organic layer underwent filtration and subsequent evaporation. The product was purified by column chromatography (Kieselgel 60) with step-wise gradient elution using hexane–EA (9 : 1), hexane–EA (30 : 10), and chloroform–methanol (10 : 1). A total of 19.7 mg, representing 48.6% of the target substance, was recovered as a colorless oil. The resulting product was dissolved in dry acetone for quaternization and treated with a 6-fold excess

of iodomethane. The synthesis yielded 22.4 mg (96%) of DOGG–Chol⁺I⁻, which appeared as white crystals. ¹H NMR (CDCl₃; m, J, 300 MHz) 0.90 (6H, s, 2H18), 1.28–1.32 (40H, m, 2H4–7; 2H12–17), 1.63 (4H, m, 2H3), 1.88 (2H, H3' (GABA)), 2.03 (8H, m, 2H8; 2H11), 2.34 (4H, 2H2), 2.43 (2H, H2' (GABA)), 3.27 (2H, m, H4'), 3.34 (2H, H1' DMAE), 4.20–4.28 (6H, dm, 2H2' (glycerol)), H2' DMAE), 4.89 (1H, H1'' (glycerol)), 5.36 (4H, m, 2H9; 2H10).

Competitive radioligand binding assay

Membranes derived from the electric organ of the *Torpedo californica* electric ray, which contain muscle-type $\alpha 1\beta 1\gamma\delta$ nAChRs (with a terminal concentration of toxin-binding sites at 0.52 nM), or cells from the GH4C1 cell line engineered to express human neuronal $\alpha 7$ nAChRs (with a final concentration of toxin-binding sites at 0.4 nM), underwent incubation with diverse concentrations of acylcholines. These were introduced as a solution in DMSO, maintained at room temperature within a binding buffer (composed of 20 mM Tris-HCl, 1 mg/mL BSA, and adjusted to pH 8.0) for 2 h 50 min or 3 h 30 min, respectively. For each specific experimental data point, including the control conditions representing 100% and 0% binding, the final buffer was modified to match the peak DMSO concentration, ranging from 0.15 to 1.5% across different experimental sets, within the reaction medium containing the acylcholines under examination. Subsequently, [¹²⁵I]- α Bgt was introduced in the samples at a final concentration of 0.2 nM, followed by an additional 5-min incubation period. Unbound [¹²⁵I]- α Bgt was eliminated from the reaction mixture via rapid filtration using GF/C filters (Whatman, UK), followed by three washes with the binding buffer (3 mL per wash). The filters used were pretreated with 0.25% polyethyleneimine. Binding was quantified as 100% under conditions where the radioligand bound without any competitors. Nonspecific binding (0%) was quantified through a comparable experiment involving the incubation of muscle and $\alpha 7$ nAChR preparations with 9 μ M α -cobratoxin (CTX) for a duration of 2 h and 50 min or 3 h and 30 min, respectively. The quantification of bound [¹²⁵I]- α Bgt was conducted employing a Wallac Wizard 1470 γ -counter (GMI Inc., USA). The efficacy of the test compounds in interacting with their respective targets was quantified through IC₅₀ value analysis, utilizing OriginPro 2015 (Microcal, USA).

Calcium imaging

Human neuroblastoma SH-SY5Y cells were cultured in a growth medium consisting of a DMEM medium ("PanEco," Russia), 10% fetal bovine serum (FBS, ne-

oFroxx GmbH, Germany), penicillin (50 IU/mL), and streptomycin (50 µg/mL, PanEco) at 37°C in a CO₂ incubator (5% CO₂). In preparation for calcium imaging, the cells were seeded into a black 96-well plate (SPL Life Sciences, South Korea) at a density of 5,000–10,000 cells per well, and cultivation proceeded until an 80–90% confluent monolayer formed. Following this, the growth medium was substituted with a buffer solution containing 140 mM NaCl, 2 mM CaCl₂, 2.8 mM KCl, 4 mM MgCl₂, 20 mM HEPES, and 10 mM glucose, adjusted to pH 7.4. The cells underwent incubation with the Fluo-4 Direct Calcium Assay Kit (ThermoFisher Scientific, USA) for 30 min in the dark at 37°C, and then for another 30 min at room temperature. Prior to the introduction of the specific α7 nAChR agonist (200 nM PNU 282987 (Tocris, UK)), the SH-SY5Y cells were incubated for 20 min with solutions comprising the test compounds and a specific α7 nAChR positive modulator (10 µM PNU 120596 (Tocris)). Complete inhibition of the observed calcium responses was achieved by incubating the cells with a 1 µM solution of α-cobratoxin (CTX), a specific antagonist of α7 nAChR, for 20 min.

A Hidex Sense Microplate Reader (Hidex, Finland) was employed to record changes in the fluorescence of the Fluo-4 dye ($\lambda_{\text{ex/em}} = 485/535 \pm 10$ nm). OriginPro 2017 (OriginLab Corporation, USA) was employed for the analysis of fluorescence intensity variations.

Rezazurin test for assessing cellular metabolic activity

SH-SY5Y cells were introduced into a transparent 96-well plate (SPL Life Sciences, Korea) at a seeding density of 3,000 cells per well. Following a 24-h incubation period, the test compounds Ol-Chol⁺I⁻, Ol-Chol⁺Mes⁻, DOGG-Chol⁺I⁻, and DOTAP⁺Mes⁻ were introduced into the cellular cultures. These compounds were administered at concentrations ranging from 6.25 to 100 µM. Concurrently, nAChR ligands, specifically 100 µM nicotine (Nic, Sigma-Aldrich, Germany), mecamylamine (Mec), d-tubocurarine (d-TC), and methyllycaconitine (MLA); 10 µM PNU 120596, PNU 282987 (Tocris, UK); and 1 µM α-cobratoxin (CTX) were also added. The cells were cultured for 72 h, followed by a determination of cellular metabolic activity using resazurin as previously described [18]. Following the removal of the culture medium, the cells were subjected to incubation for a duration of 4 h in the presence of a resazurin solution (Macklin Inc., China) prepared in a buffer formulated with 140 mM NaCl, 2 mM CaCl₂, 2.8 mM KCl, 4 mM MgCl₂, 20 mM HEPES, 10 mM glucose, and a pH of 7.4. Thereafter, the fluorescence intensity ($\lambda_{\text{ex/em}} = 550/590 \pm 10$ nm) of

the resazurin reduction product (resorufin) was measured using a microplate fluorometer (Hidex, Finland). Wells without cells served as negative controls, while intact SH-SY5Y cells served as positive controls.

Determination of extracellular lactate dehydrogenase (LDH) activity to assess the cytotoxicity of the compounds

SH-SY5Y cells were seeded into a transparent 96-well plate (SPL Life Sciences, Korea) at a density of 3,000 cells per well. Following a 24-h incubation period, the cells were subjected to treatment with Ol-Chol⁺I⁻ and DOTAP⁺Mes⁻ at concentrations of 6.25–100 µM. After an additional 72 h of culture, extracellular lactate dehydrogenase activity was assessed via the LDH Cytotoxicity Assay kit (Wuhan Servicebio Technology Co., Ltd., China), following the manufacturer's protocol. Following a 30-min incubation period of an 80-µL aliquot of the selected culture medium with 80 µL of a working solution for lactate dehydrogenase detection, the optical density of the solution was assessed. This measurement was taken at $\lambda = 490$ nm using a Hidex Sense Microplate Reader (Hidex, Finland). Wells without cells served as negative controls, and lysed SH-SY5Y cells (Cell lysis buffer, LDH Cytotoxicity Assay kit) served as positive controls.

RESULTS AND DISCUSSION

Synthesis of acylecholines and their cationic dioleoyl analogs

The compounds synthesized via chemical means and utilized in this study are shown in *Fig. 1*. The synthesis of oleoylcholine in the form of iodide (Ol-Chol⁺I⁻) and mesylate (Ol-Chol⁺Mes⁻) was conducted as described previously [19]. DOTAP mesylate was synthesized from 1,2-dimethylaminopropanediol-1,2 and oleic acid [20], followed by quaternization with dimethyl sulfate. DOGG-Chol⁺I⁻ was obtained from 1,2-dioleoylglycerol by the sequential introduction of GABA and DMAE into its structure followed by quaternization with iodometane.

Interaction of the synthesized compounds with nicotinic acetylcholine receptors

The capacity of the synthesized compounds to interact with the orthosteric binding site of nAChR was evaluated using a radioligand binding assay, and the functional activity of nAChR was determined by calcium imaging.

To elucidate the interaction of iodide and mesylate oleoylcholine, alongside the cationic lipids DOGG-Chol⁺I⁻ and DOTAP⁺Mes⁻, with the orthosteric

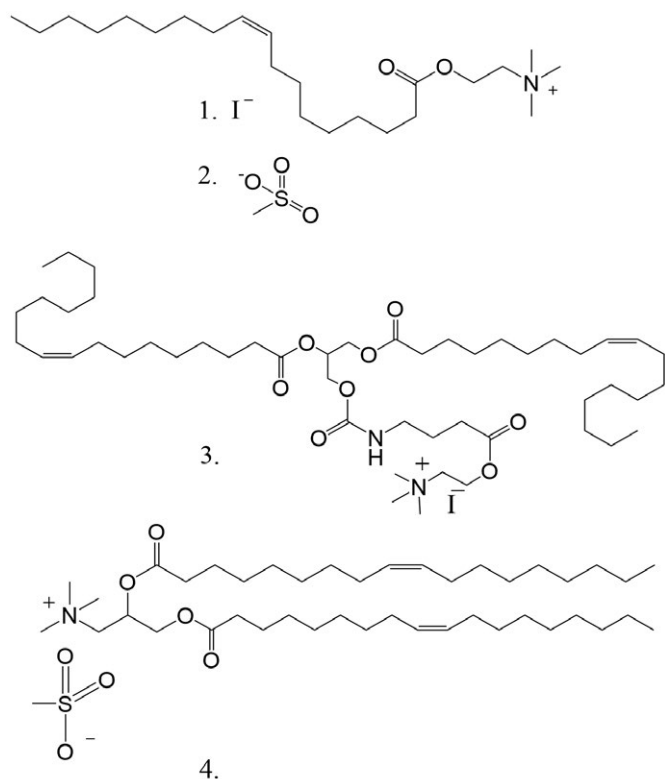


Fig. 1. The structures of the acylcholines under study: (1) oleoylcholine iodide (OI-Chol⁺I⁻), (2) oleoylcholine mesylate (OI-Chol⁺Mes⁻), (3) 4-(((1,3-bis(oleoyloxy)propan-2-yl)oxy)carbonyl)amino)butanoic acid choline ester iodide (DOGG-Chol⁺I⁻), (4) 1,2-Bis-(9Z-octadecenoyl)-3-trimethylammonium 1,2-dihydroxypropane mesylate (DOTAP⁺Mes⁻)

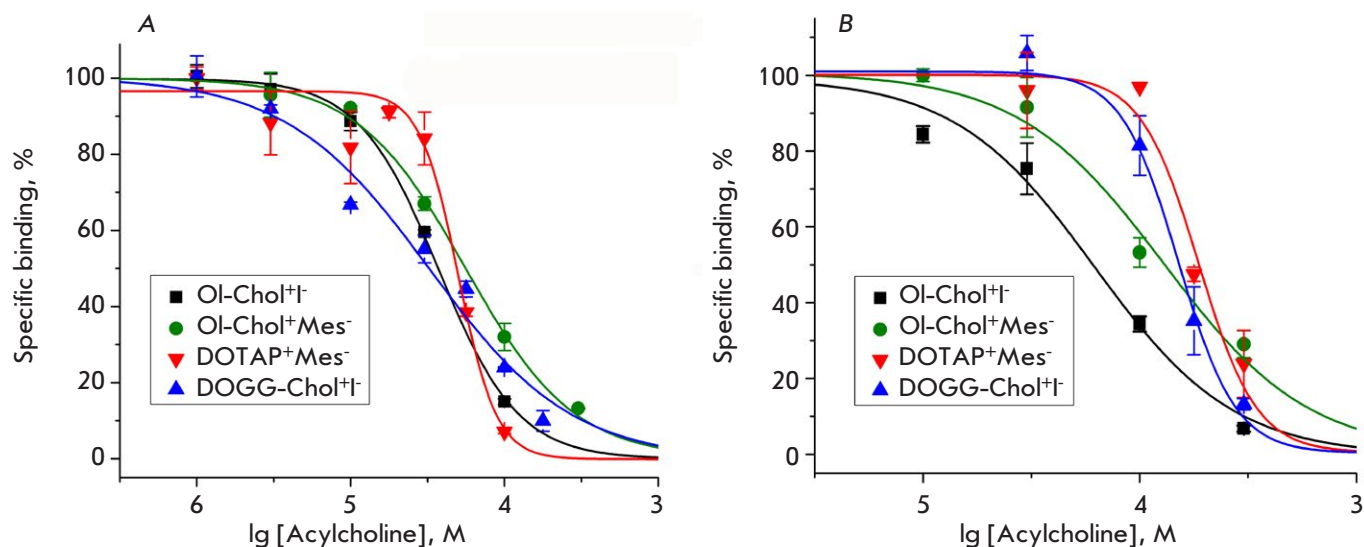


Fig. 2. The dependence of the specific binding of radioactive [¹²⁵I]-αBgt to the muscle nAChR of the ray *Torpedo californica* (A) and human α7 nAChR (B) on the concentration of the acylcholines under study and their dioleoyl analogs. Each data point on the graphs represents the mean ± standard error of the mean, $n = 2-3$

binding site of nAChR, we examined their competitive binding with [¹²⁵I]-α-bungarotoxin ([¹²⁵I]-αBgt) to the muscle nAChR from the electric organ of *T. californica* ray and to a human neuronal α7 nAChR via a radioligand binding assay (Fig. 2).

All the compounds were found to bind to the orthosteric sites of both receptors (Fig. 2). The affinity of OI-Chol⁺I⁻ and OI-Chol⁺Mes⁻ and that of the dioleoyl analogs DOGG-Chol⁺I⁻ and DOTAP⁺Mes⁻ to the muscle-type receptors (IC₅₀ range = 32.5–56 μM) was found to be 2.0–5.5 times higher than that to human α7 nAChRs (IC₅₀ range = 62–178 μM) (Table 1). Concurrently, oleoylcholine iodide demonstrated a twofold efficacy in interacting with the orthosteric binding site of muscle and α7 nAChRs (IC₅₀ = 37 and 62 μM, respectively) compared to the mesylate form (IC₅₀ = 56 and 129 μM, respectively) (Table 1).

The binding affinity of the novel acylcholine analogs (OI-Chol⁺Mes⁻, DOGG-Chol⁺I⁻, DOTAP⁺Mes⁻) for the muscle-type nicotinic acetylcholine receptor from the electric organ of *T. californica* was established via a radioligand binding assay, yielding IC₅₀ values from 32.5 to 56 μM (Fig. 2 and Table 1). This affinity was found to be comparable to that of previously investigated endogenous acylcholines (arachidonoylcholine, oleoylcholine, linolenoylcholine, and docosahexaenoylcholine), which exhibited IC₅₀ values ranging from 18.7 to 93 μM [5]. It is of interest that the novel compounds displayed lesser affinity (129–178 μM) for the human neuronal α7 subtype of nAChR compared to prior studies (14.2–80 μM) [5]. Our study is the first to demonstrate the effect of the nature of the coun-

terion of a given acylcholine on the efficiency of its interaction with the receptor: oleoylcholine iodide exhibited approximately a twofold affinity for its mesylate (Table 1).

Radioligand binding assays demonstrated that all the acetylcholine analogs under study bind to the $\alpha 7$ nAChR. Subsequently, calcium imaging was employed to examine their capacity to suppress the functional reactivity of this specific receptor subtype. For this purpose, SH-SY5Y neuroblastoma cells were used, which endogenously express $\alpha 7$ and several other nAChR subtypes [21]. All tested compounds were found to inhibit cellular responses induced by $\alpha 7$ nAChR activation with PNU282987, a specific agonist of this receptor, in the presence of the positive allosteric modulator PNU120596 (Fig. 3). Oleoylcholine mesylate was identified as the most potent inhibitor, with an IC_{50} value of $0.79 \mu\text{M}$. Its effectiveness dropped fivefold when administered as iodide (Fig. 3, Table 1). DOGG-Chol⁺I⁻ and DOTAP⁺Mes⁻ were 9- and 19-fold less effective than Ol-Chol⁺Mes⁻, respectively (Fig. 3, Table 1).

This study provides the initial evidence of a substantial discrepancy in how the salt form of oleoylcholine modulates its inhibitory effect on nAChR. Oleoylcholine mesylate demonstrated the highest affinity for $\alpha 7$ nAChR and presented a significantly flatter calcium response curve (Fig. 3, Table 1). Consequently, this compound demonstrated efficacy even at submicromolar concentrations ($IC_{20} \sim 0.1 \mu\text{M}$, $IC_{50} = 0.8 \mu\text{M}$). At the same time, the other compounds investigated in the present study (Ol-Chol⁺I⁻, DOGG-Chol⁺I⁻, DOTAP⁺Mes⁻) and in our previously published work (iodides of arachidonoylcholine, linolenoylcholine, and docosahexaenoylcholine) were active only in the micromolar concentration range (2–15 μM) (Table 1) [5].

Assessing the interaction of substances with the orthosteric ligand-binding site via a radioligand binding assay demonstrated that the affinity of all eval-

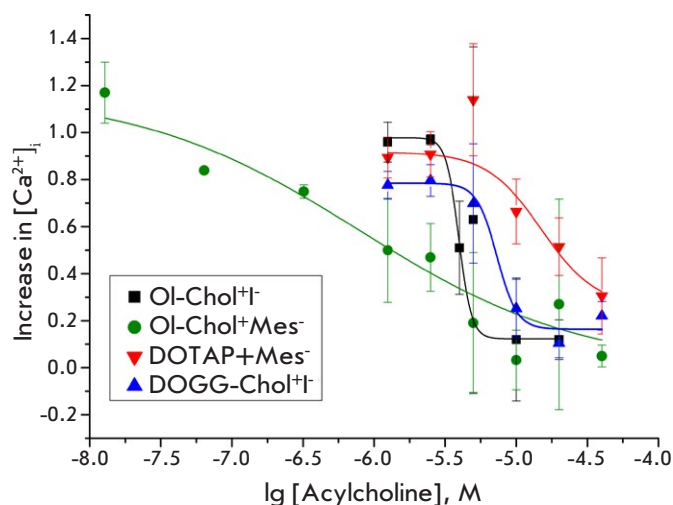


Fig. 3. Inhibition by acylcholine analogs of the calcium responses induced by the activation of $\alpha 7$ nAChR with the specific agonist PNU282987 (200 nM) in SH-SY5Y neuroblastoma cells. The calculated inhibition parameters (IC_{50}) are shown in Table 1. The data are presented as mean \pm standard deviation, $n = 4-6$

uated compounds for $\alpha 7$ nAChR was considerably lower than that determined by functional inhibition, as observed in calcium imaging (Table 1). This finding suggests the potential involvement of alternative molecular mechanisms in the inhibitory action of acylcholine analogs, possibly through interaction with allosteric binding sites on the receptor. It is noteworthy that increasing the complexity of the lipophilic moiety of oleoylcholine analogs (DOGG-Chol⁺I⁻ and DOTAP⁺Mes⁻) by introducing an additional oleic acid residue resulted in a decrease in the affinity of these compounds for the receptor. This reduction was evident in both radioligand binding assays (Fig. 2, Table 1) and calcium imaging experiments (Fig. 3, Table 1). The structural complexity of the receptor could potentially impede ligand interaction.

Table 1. The affinity of acylcholine analogs for two nAChR subtypes, presented as IC_{50} values (μM), calculated basing on a radioligand binding assay and Ca^{2+} imaging data

Acylcholines	nAChR <i>T. californica</i>		$\alpha 7$ nAChR	
	Radioligand binding assay, IC_{50} (μM)		Radioligand binding assay, IC_{50} (μM)	Ca^{2+} imaging, IC_{50} (μM)
Ol-Chol ⁺ I ⁻	37 ± 1		62 ± 4	3.95 ± 0.18
Ol-Chol ⁺ Mes ⁻	56 ± 2		129 ± 8	0.79 ± 2.24
DOGG-Chol ⁺ I ⁻	50 ± 3		151 ± 4	7.32 ± 2.97
DOTAP ⁺ Mes ⁻	32.5 ± 3.5		178 ± 8	14.99 ± 9.10

The effect of oleoylcholine and its dioleoyl analogs on the viability and proliferative activity of SH-SY5Y neuroblastoma cells

Several minutes are sufficient to assess the functional activity of oleoylcholine and its dioleoyl analogs with respect to $\alpha 7$ nAChR. However, the targeted intracellular delivery of nucleic acids and similar compounds requires the incubation time of cationic lipids with cells to be extended to several hours or even days. In this regard, we conducted a comprehensive evaluation of the long-term effects (over 72 h) of oleoylcholine and its analogs DOGG-Chol⁺I⁻ and DOTAP⁺Mes⁻ on the viability and proliferation of SH-SY5Y neuroblastoma cells. This investigation included a thorough examination of the dependence of the observed effects on the activation or inhibition of nAChR using known agonists, a positive allosteric modulator, and antagonists. For this purpose, the cellular metabolic activity was examined through the resazurin assay, and extracellular lactate dehydrogenase activity was measured to assess the compound-induced cytotoxicity. Our findings demonstrated that oleoylcholine (6.25–100 μ M) did not elicit a significant alteration in cell viability (as depicted in Fig. 4A,B). Conversely, the compound DOGG-Chol⁺I⁻ demonstrated cytotoxic effects at all the concentrations tested (13–28% cell death, IC₅₀ > 100 μ M (Fig. 4A)). The cytotoxic effect of the cationic lipid DOTAP⁺Mes⁻ exhibited a pronounced

concentration-dependence (IC₅₀ = 26.18 \pm 0.16 μ M in the resazurin assay (Fig. 4A) and 19.28 \pm 5.64 μ M in the LDH assay (Fig. 4B)).

Our prior findings [5] indicated that A549 lung adenocarcinoma cells treated with oleoylcholine iodide (100 μ M, 24 h) exhibited a concentration-dependent decrease in viability as a consequence of induced apoptosis, achieving a 45% rate. Although these cells demonstrate the presence of $\alpha 7$ nicotinic acetylcholine receptors [22], it is unlikely that this receptor subtype is responsible for the observed effect. This conclusion is supported by the finding that methyllycaconitine (10 μ M), a specific inhibitor, did not alter the cytotoxic action of oleoylcholine [5].

This investigation sought to determine if the enduring impact of the examined cationic lipids on SH-SY5Y neuroblastoma cell viability is attributable to their interaction with nAChRs. With this objective in mind, SH-SY5Y cells were subjected to incubation for 72 h with the lipids under investigation at two distinct concentrations below their IC₅₀ for cytotoxic activity (10 and 50 μ M for OI-Chol⁺I⁻, OI-Chol⁺Mes⁻, DOGG-Chol⁺I⁻; 5 and 25 μ M for DOTAP⁺Mes⁻), accompanied by the introduction of specific nAChR ligands (Fig. 5). SH-SY5Y cells have been observed to express homopentameric $\alpha 7$ nAChR, as well as various heteropentameric nAChRs formed by combinations of $\alpha 3$ -, $\alpha 5$ -, $\beta 2$ -, and $\beta 4$ -subunits [21]. The ex-

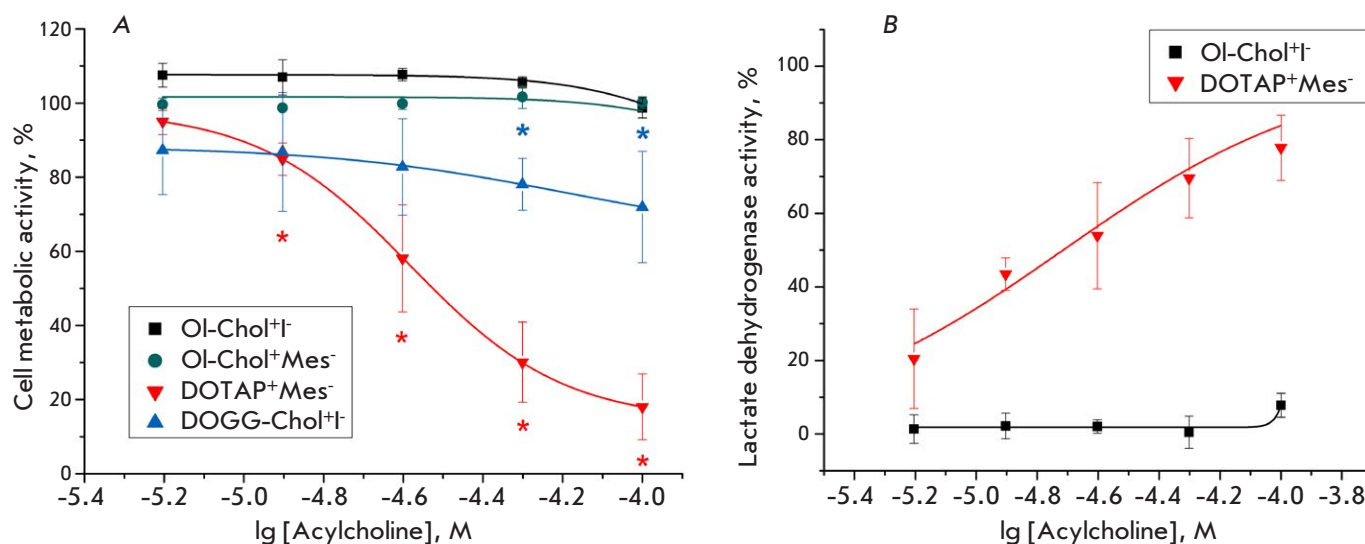


Fig. 4. The effect of OI-Chol⁺I⁻, OI-Chol⁺Mes⁻, DOGG-Chol⁺I⁻ and DOTAP⁺Mes⁻ on the viability and proliferative activity of SH-SY5Y cells. The cells were incubated with various concentrations of the substances for 72 h. (A) the cellular metabolic activity was determined using the resazurin test; (B) the cytotoxicity of DOTAP⁺Mes⁻ and OI-Chol⁺I⁻ was determined by quantifying extracellular LDH activity. The data are presented as mean \pm standard deviation, $n = 3$. * $p < 0.05$ compared with control values, Mann–Whitney U-test

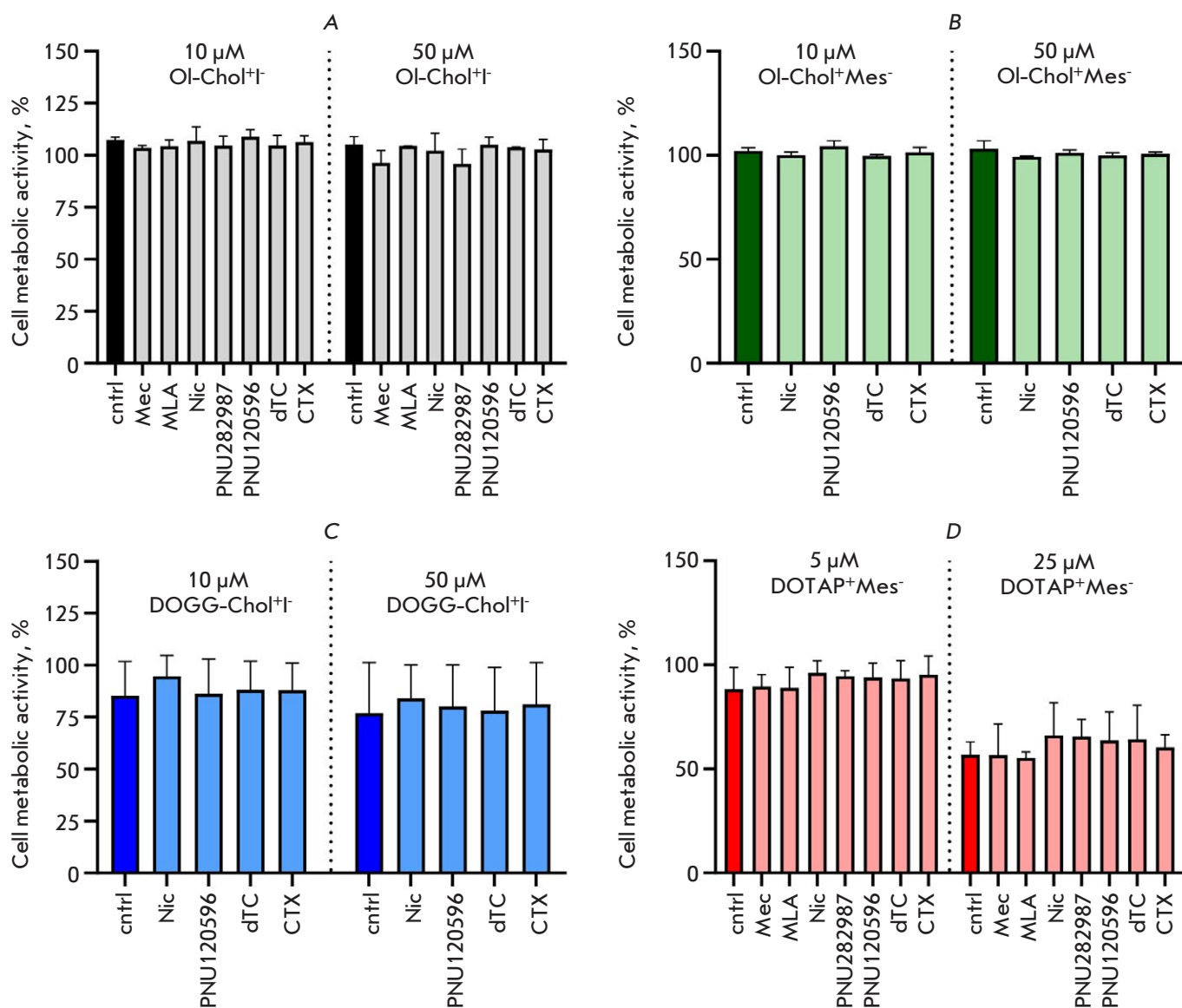


Fig. 5. The effect of nAChR ligands on the long-term effects on the metabolic activity of SH-SY5Y cells over 72 h (A) OI-Chol⁺I⁻, (B) OI-Chol⁺Mes⁻, (C) DOGG-Chol⁺I⁻, and (D) DOTAP⁺Mes⁻. The nAChR ligands employed were: nicotine (Nic), mecamylamine (Mec), d-tubocurarine (d-TC), and methyllycaconitine (MLA) at a concentration of 100 μ M; PNU120596 and PNU282987 at a concentration of 10 μ M; and α -cobratoxin (CTX) at a concentration of 1 μ M. The data are presented in relation to the metabolic activity values of intact SH-SY5Y cells. Cntrl is the relative metabolic activity of cells in the presence of the indicated concentration of (A) OI-Chol⁺I⁻, (B) OI-Chol⁺Mes⁻, (C) DOGG-Chol⁺I⁻, and (D) DOTAP⁺Mes⁻. The data are presented as the mean \pm standard deviation, $n = 3$. * $p < 0.05$, Mann–Whitney U test, compared with control values

perimental findings demonstrate that non-selective agonists (nicotine), antagonists (mecamylamine and d-tubocurarine), and specific $\alpha 7$ nAChR ligands (e.g., the agonist PNU282987, the positive allosteric modulator PNU120596, and antagonists such as methyllycaconitine and α -cobratoxin) did not alter the effects of the examined compounds on SH-SY5Y cells (Fig. 5).

CONCLUSION

Acylated cholines represent a recently discovered class of endogenous fatty acid analogs of acetylcholine which remain poorly studied. Our previous research demonstrated that choline esters of unsaturated fatty acids function as modulators of the acetylcholine system. The present work utilizes

oleoylcholine, which earlier demonstrated the most significant activity in inhibiting $\alpha 7$ nAChR, to provide the first evidence that the counterion of the quaternary ammonium group substantially impacts the compound's capacity to interact with nAChR. According to radioligand analysis of [125 I]- α Bgt binding to the orthosteric binding site of nAChR, oleoylcholine iodide interacted with muscle and neuronal $\alpha 7$ nAChR twice as effectively as mesylate. These results suggest that, under biological experimental conditions in the working buffer solution, the counterion is not replaced by chloride despite its excess. Furthermore, the size of the counterion appears to significantly influence the interaction of acylcholine with the orthosteric acetylcholine binding site on the receptor. Utilizing calcium imaging, a method that captures the functional response of cells to nAChR activation, it was demonstrated that both salt forms of oleoylcholine (iodide and mesylate) were significantly more potent inhibitors of $\alpha 7$ nAChR ($IC_{50} = 3.95$ and $0.79 \mu M$, respectively) than was observed in experiments involving competition with [125 I]- α Bgt. In this case, the enhanced inhibitory activity of oleoylcholine derivatives can be postulated to be associated with their additional interaction with allosteric binding sites on the receptor molecule, which is not detected by a radioligand-based analysis.

A significant finding of this study is the discovery that DOTAP, a cationic lipid widely used in lipid nanoparticles for the delivery of nucleic acids inside mammalian cells, possesses the capacity to inhibit both muscle-type and neuronal-type nAChRs. The potential of DOTAP, a cationic lipid, to modify acetylcholine receptor activity, though less pronounced than that of oleoylcholine, should be considered in the design of medications incorporating this compound. In contrast to oleoylcholine, the lipophilic component of DOTAP features two oleic acid residues. In the present study, an oleoylcholine analog was synthesized in

which the lipophilic moiety also contained two oleic acid residues within the 1,2-diacylglycerol backbone. The choline group was separated from the lipophilic moiety by a GABA-based linker (DOGG-Chol $^+I^-$). Similar to DOTAP, this analog demonstrated affinity for $\alpha 7$ nAChR, as substantiated by radioligand binding assays and functional responses in calcium imaging experiments. Nevertheless, its activity was considerably less pronounced than that of oleoylcholine. Whereas oleoylcholine fosters the proliferation of SH-SY5Y neuroblastoma cells, DOGG-Chol $^+I^-$ and DOTAP $^+Mes^-$ induced a significant cytotoxic effect on these cells. This effect, however, was not mediated by interaction with nAChRs, since specific nAChR ligands were unable to block it.

In summary, the choline group common to all the acylcholines under study makes them act as nAChR inhibitors. Conversely, the modification of the lipophilic domain of the molecule with an additional oleic acid group has been shown to attenuate its inhibitory function while amplifying its cytotoxic action, a process that occurs through a mechanism independent of nAChRs. ●

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