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New Type of BACE1 siRNA Delivery to Cells

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Background: Small interfering RNA (siRNA) gene therapy is a new molecular approach in the search for an efficient therapy for Alzheimer disease (AD), based on the principle of RNA interference. Reducing BACE activity can have great therapeutic potential for the treatment of AD. In this study, receptor-mediated delivery was used to deliver opioid peptide-conjugated BACE 1 to INR-32 human neuroblastoma cells.





Material/Methods: An INR-32 human neuroblastoma cell line was stably transfected to express the APP cDNA coding fragment containing the predicted sites for cleavage by α , β , or γ -secretase. This was then treated with BACE 1 siRNA to silence BACE gene expression. BACE gene transcription and translation was determined using BACE-1 siRNA cross-linked with opioid peptide, together with RT-PCR, Western blot analysis, and ELISA.

Results: Receptor-mediated delivery was used to introduce BACE1 siRNA to the APP – INR 32 human neuroblastoma cells. Decreased BACE mRNA and protein expression were observed after the cells were transfected with BACE1 siRNA.

Conclusions: Delivery of BACE1 siRNA appears to specifically reduce the cleavage of APP by inhibiting BACE1 activity.

MeSH Keywords: **Amyloid beta-Peptides • Gene Expression • RNA, Small Interfering**

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Background

In many countries, the prevalence of neurodegenerative diseases has increased as the population has aged, and finding a treatment or cure has become the focus of many researchers worldwide. Alzheimer disease is the most common cause of dementia, accounting for approximately 70% of cases in subjects aged over 70. Between 18 and 24 million people have AD worldwide, two-thirds of whom are living in developed or developing countries [1–3].

Alzheimer disease (AD) is a chronic neurodegenerative disorder characterized by the neuropathological conclusion of intracellular neurofibrillary tangles and extracellular amyloid plaque accumulation in vulnerable regions of the brain [4]. The accumulation of amyloid- β peptides ($A\beta$) plays a key role in AD pathogenesis [5–7]. The proteolytic cleavage of APP (amyloid precursor protein) occurs through secretase activity, and $A\beta$ is obtained by sequential cleavage of β and γ secretase [8]. β -secretase cleavage followed by γ -secretase leads to the formation of either $A\beta_{40}$ or $A\beta_{42}$, which together form insoluble amyloid plaques. β -secretase (BACE1-Beat amyloid-cleaving enzyme-1) is the initial and rate-limiting enzyme involved in $A\beta$ production [9]. Hence, strategies to reduce BACE1 activity may have therapeutic potential for the treatment of AD [10]; a particularly promising approach being the use of small interfering RNAs (siRNAs) to suppress BACE1 [11,12].

Although the highly efficient and specific delivery of therapeutically important molecules into cells is one of the most intensely pursued areas in biomedical research, frustratingly little progress has been made over the last several decades. While a number of powerful new therapeutic strategies have been introduced in the last few years, shortcomings in the mode of delivery have prevented them from reaching their potential [13–18]. However, one potentially valuable route of enquiry for drug delivery is derived from the observation that most hormones and growth factor ligands are naturally endocytosed after binding to their cognate cell surface receptors and transport to the cytoplasm and the nucleus. Based on this approach, a combination of protein engineering, chemistry, and cell biology may be used to develop a highly efficient delivery tool that circumvents the limitations of currently-used approaches. Morphine and other alkaloid opiates are the most effective analgesics and are commonly used to treat moderate to severe pain. Alkaloid opiates produce analgesic responses by activating opioid receptors, particularly μ -type receptors (MOR), in the central nervous system [19,20]. Opioid receptors and their ligands have been identified mostly in the central nervous system, but are also known to occur in peripheral organs and tissues [21,22]. Recently, the presence of opioid peptides and receptors was reported in various cancer cell types. The opioid receptors were shown to be integral components

of a wide variety of malignant and benign human and animal tumor cells of ectodermal, mesodermal, and endodermal origin in neural and non-neural tissues.

The aim of the study was to evaluate the potential of small interfering RNAs (siRNAs) bound to opioid peptide to suppress BACE1 activity and deliver bioactive molecules to cells through the opioid receptor internalization mechanism.

Material and Methods

Reagents

The INR-32 human neuroblastoma cell line was obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany). All standard cell culture reagents were obtained from Gibco-BRL (Bethesda MD, USA). TRIzol reagent was obtained from Invitrogen Life Technologies Inc. (Rockville MD, USA). Primer pairs were designed using PrimerExpress software (Applied Biosystems, Foster City, CA, USA) and purchased from Integrated DNA Technologies Inc. (Coralville, IN, USA)

Cell culture

The INR-32 human neuroblastoma cells obtained from the ATCC Company (American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle's Minimum Essential Medium (EMEM-GIBCO BRL, Invitrogen, Carlsbad, CA, USA). The basic formulation was supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μ g/ml) and 10% heat-inactivated fetal bovine serum.

Peptide synthesis

Peptides (Tyr-Pro-Trp-Phe-Cys-NH₂) were prepared by manual solid-phase synthesis, using Rink Amide resin and Fmoc (Sigma Aldrich St Louis, MO, USA) protection, as described by Fichna et al. [23]. After cleavage from resin by TFA/TIS treatment (EMD Milipore Corp. Billerica, MA, USA), the crude peptides were purified by preparative RP HPLC (Agilent Technologies, Foster City, CA, USA). The purity of synthesized peptides was established by analytical RP HPLC.

Construction of the pCI-neoAPP vector

The TRIzol RNA extraction reagent (Invitrogen Life Technologies) was used to extract total RNA from the brain of a patient with AD. Five micrograms of isolated RNA was reverse-transcribed at 42°C for 60 min using the ImProm-II™ Reverse Transcription System Kit (Promega, Madison WI, USA). The obtained cDNA was used to amplify the APP fragment coding

the 240 C-terminal amino acids of human APP, which contains the predicted sites for cleavage by α , β , or γ -secretase. The APP fragment cDNA was amplified using an Expand High Fidelity PCR Kit (Roche Molecular Biochemicals Germany) and primers: 5' TTTGGATCCATGACACACCTCCGTGTGATTTATGAGCG 3', 5' TTCTCGAGCTAGTCTGTCATCTGCTCAAAGAAGCTTGTAGG 3' containing restriction sites for BamH1 and XhoI. The amplification fragment was cloned into a pTX-FLAG expression system, resulting in a high level of APP expression in bacterial cells. The APP-fragment was then recloned to the pCI-neo (Promega, Madison WI) mammalian expression system. The pCI-neo APP recombinant plasmid was used for transfection of the INR-32 human neuroblastoma cell line using lipofectamine reagent (Gibco-BRL, Bethesda MD., USA). Stable APP expression was obtained by selecting transfected cells with the antibiotic G418. A transfected INR-32 human neuroblastoma cell line with confirmed APP expression was then used as a model to study BACE1 expression and BACE1-siRNA selection.

Opioid-siRNA construction

The Blast-siRNA Target Finder program was used to evaluate the ability of siRNA to inhibit the expression of BACE1. Twenty siRNA candidate targets were obtained after homology filtering. Using Blast and Multiple sequence alignment, 5 candidate targets were analyzed and siRNA were selected. The activity level of the selected five siRNA were checked on transfected INR-32 human neuroblastoma cells. Unmodified and modified sequences of siRNA to BACE1 mRNA were synthesized by Thermo Scientific Pharmacocon RNAi Technologies (Denver, CO, USA). The selected siRNA sequences were modified into opioid-siRNA constructions. The oligonucleotides were modified with a thiol group at the 3' end. The inhibitory effect of selected siRNAs was confirmed by real-time RT-PCR on the transcriptional level, and Western blot and ELISA on the protein level. The siRNAs were selected. Peptide (Tyr-Pro-Trp-Phe-Cys-NH₂) was crosslinked with siRNA-3 using the SPDP reagent (sulfo-succinimidyl 6-[3' (2-pyridyldithio)- propionamido] hexanoate) obtained from Thermo Scientific (Rockford, IL, USA) according to the manufacturer's protocol. The opioid-siRNA construct was then used to transfect APP INR-32 human neuroblastoma cells.

Incubation with opioids and opioid-siRNA

The INR-32 human neuroblastoma cells were seeded in 25-ml cell culture flasks in standard growth medium at a density of 2.5×10^5 cells/flask (60–80% confluent). After 24 h, growth medium was replaced by fresh growth medium supplemented with the opioid-siRNA construction at a concentration of 10^{-7} mol/L. The effect of 10^{-7} mol/l opioids and opioid-siRNA complex on the expression of BACE1 mRNA was investigated after 12 and 24 h. Cells incubated without the opioid-siRNA mixture were used as a control. After incubation, the cells

were washed twice with EMEM medium to remove the added opioids, and then harvested by trypsinization. Transfection of BACE1 siRNA (75 pmoles) and the control siRNA duplex was performed with Oligofectamin reagent and incubated for 5–7 hours. Following this, 1 ml of EMEM medium was added without removing the transfection mixture, and the culture was incubated for an additional 24 h. After incubation, the media were aspirated and replaced with normal medium and incubated for 24 h at 37°C. The cells were frozen and kept at –80°C for further experiments.

Receptor binding assay

The μ -opioid receptor-binding studies with the opioid peptide and opioid-BACE1 siRNA were performed according to a previously described method, with some modifications [24]. Briefly, crude membrane preparations isolated from Wistar rat brains were incubated at 25°C for 120 min with an appropriate concentration of a tested peptide in the presence of 0.5 nM [³H]DAMGO in a total volume of 0.5 mL of 50 mM Tris-HCl (pH 7.4) containing MgCl₂ (5 mM), ethylenediaminetetraacetic acid (1 mM), and bacitracin (20 mg/L). Incubation was terminated by rapid filtration through Whatman GF/B (Brentford, UK) glass fiber strips, which had been presoaked for 2 h in 0.5% polyethylamine, using a Sampling Manifold (Millipore, Billerica, MA, USA). The filters were washed 3 times with 4 mL of ice-cold Tris buffer solution. The bound radioactivity was measured with a Tri-Carb 2100 TR liquid scintillation counter (Packard, Ramsey, MN, USA) after overnight extraction of the filters in 4 mL of Ultima Gold scintillation fluid (Perkin-Elmer, Wellesley, MA, USA).

Three independent experiments for each assay were carried out in duplicate.

Quantitative real-time RT-PCR

Total RNA was extracted from control and transformed INR-32 human neuroblastoma cells using the TRIZOL RNA extraction reagent (Invitrogen Life Technologies), according to the standard acid guanidinium phenol-chloroform method [25]. The extracted RNA was analyzed by agarose gel electrophoresis and only cases with preserved 28S, 18S, and 5S ribosomal RNA bands, indicating good RNA quality, were used in the study. Total RNA was digested with DNase (GIBCO) at room temperature for 15 min. The amount of purified RNA was determined using spectrophotometry at 260 nm in a Nanodrop analyser (ND-100; Nanodrop Technologies, Wilmington, DE, USA). The 260 nm: 280 nm ratio was measured to verify the purity of the sample, with values between 1.8 and 2.1 indicating that the quality of the RNA obtained was optimal and suitable for quantitative real-time PCR (qRT-PCR). Five micrograms of digested RNA were reverse-transcribed at 42°C for 60

min in a total reaction volume of 20 ml using the ImProm-II™ Reverse Transcription System kit (Promega, Madison WI, USA). Obtained cDNA was used in real-time PCR reaction.

Real-time PCR based on TaqMan™ technology was performed using a master mix prepared according to the FastStart Universal Probe Master (ROX) from Roche Applied Science. Probes and primers were designed using the online *Universal Probe Library* (www.universalprobelibrary.com). Primer sequences and probe numbers are as follows: MOR (forward, 5'-GTCCTTTATGCATTCTGGATGAAAAC-3', reverse, 5'-CTGGAAGCAG AAAGTCTCCGTTG-3, probe: #3), DOR (forward 5' GGCTACGCCAATA GCAGCCTCAAC 3' reverse 5' CCCGTCCGATGGTCCCGCGGTTGGCG 3', probe #21) KOR (forward 5' GCTGCTCTCTCCAGCTATTACTTCT 3', probe # 26), BACE 1 (forward 5'TTTGTGGAGATGGTG GACAA3', reverse 5'CAGCACCCACTGCAAAGTTA3' probe: #16), APP (forward 5'GCTGCTGAACCCAGAT3', reverse 5'CCCACTCCCATTG TGGACAT3' PROBE # 19) and GAPDH (forward, 5-AGCCACATCGCTCAGACAC-3', reverse, 5-GCCCAATACGACCAAATCC-3, probe: #60), which was used as an internal control for real-time PCR.

Real-time PCR was carried out in a final volume of 50 µl, with 0.05 µg cDNA, 25 µl FastStart Universal Probe Master (ROX) 2x, 250 nM probe, and 1 µM of each primer. Amplification was performed for 10 min at 95°C to activate FastStart Taq DNA polymerase and 40 rounds of 15 s at 95°C and 1 min at 60°C for amplification and signal analysis. The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to detect amplifications. Each sample was assayed in triplicate in independent reactions. Real-time PCR data was automatically calculated with a data analysis module. The results were analyzed according to the $2^{-\Delta\Delta Ct}$ method [26,27]. PCR efficiency was validated with a standard curve.

Western blot analysis

The MOR, APP, BACE1, and tubulin protein level in INR-32 human neuroblastoma cells was quantified in Western blot experiments using whole cell preparations. Protein extracts from the INR-32 human neuroblastoma cells (20–80 µg) were separated by 7.5% SDS-PAGE and transferred to a Immobilon PVDF membrane (Millipore, Billerica, Mass., USA) in 20 mmol/l Tris-HCl containing glycine (192 mmol/L) and methanol (20%) at 30V overnight. Nonspecific binding was blocked by 5% non-fat dry milk for 2 h at room temperature. The membranes were incubated with anti-MOR-1 rabbit polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz CA, USA) at a dilution of 1:1000, anti-APP rabbit monoclonal antibody (Chemicon international, Temecula CA) at 1:500, anti-BACE1 rabbit polyclonal antibody (Biocompare San Francisco, CA, USA), anti- α -tubulin rabbit monoclonal antibody (Sigma ST Louis), MO) at 1:20000,

and were subsequently probed using a One-Step Western blot kit for rabbit primary antibody (GeneScript Corp., Piscataway, NJ, USA) according to the manufacturer's protocol. The blots were developed with a Chemiluminescent HRP Substrate Kit (L00221V60, GeneScript Corp). The quantification of MOR, APP, BACE1, and tubulin content was performed by densitometric scanning of immunoblots using an Image Master VDS system (Pharmacia LKB, Freiburg, Germany). An Unstained Protein Weight Marker (Fermentas International Inc., Burlington. Ont., Canada) was used as a molecular mass marker.

Determination of cellular BACE1 and amyloid A β level using Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of secreted A β in the INR-32 human neuroblastoma cells were quantified by ELISA. The concentration of A β_{40} and A β_{42} was detected and quantified in triplicate using the β -Amyloid 1-40 or 1-42 Colometric ELISA Kit (Biosource International, Camarillo, CA) and the A β (1-40 and 1-42) ELISA assay kit (Immuno-Biological Laboratories, Gumna, Japan) according to the manufacturer's instructions. The presented level of A β was the mean result of 3 independent experiments performed on separate days. For evaluating the quantitative BACE1 levels, a BACE1 ELISA kit from FIVEphoton Biochemicals (San Diego, CA, USA) was used.

Samples were pre-coated and incubated with a polyclonal anti-BACE1 capture antibody. A second goat anti-BACE1-HRP conjugate detection antibody was added, followed by incubation and wash. Chromogen solutions A and B were added, resulting in a color change to blue. The color development was stopped and the intensity of the color was measured at 450 nm by the Thermo Labsystems Multiscan Ascent 354 (Lab recyclers).

BACE1 activity in the INR-32 human neuroblastoma cells was measured with the β Secretase (BACE1) Activity Assay Kit. A fluorometer was used to measure baseline fluorescence according to the manufacturer's instructions: excitation 320 nm, emission 405 nm.

Statistical analysis

Statistical analyses were performed using Prism 4.0 (GraphPad software Inc., San Diego CA, USA). The data are expressed as means \pm SD. Differences between groups were assessed by one-way ANOVA followed by a post-hoc multiple comparison Student-Newman-Keuls test. Scores of $p=0.05$ or lower were considered statistically significant. For the general intergroup comparison of continuous measurements, one-way analysis of variance was used if the hypotheses could be met and Kruskal-Wallis test was used if the hypotheses could not be met. Paired comparisons of the groups were done using Bonferroni or Scheffe tests.

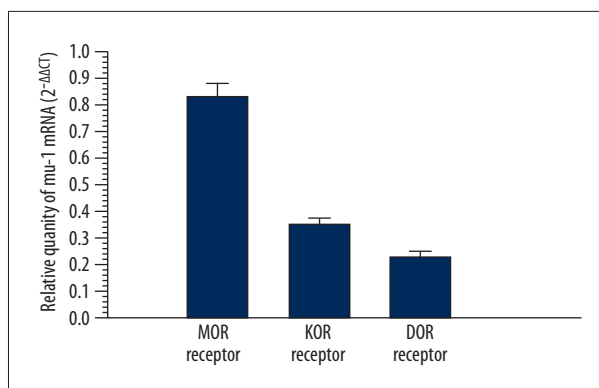


Figure 1. Expression of MOR, DOR, and KOR mRNA in INR-32 human neuroblastoma cells according to quantitative real-time RT-PCR analysis. Data are presented as mean \pm SD of 3 independent experiments performed in duplicate. Statistical significance was determined by *t* test. Differences between MOR, KOR, and DOR gene expression are statistically significant ($p < 0.001$).

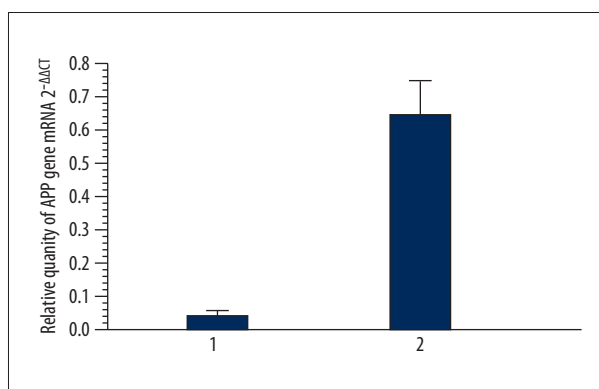


Figure 2. Expression of APP fragment mRNA in INR-32 human neuroblastoma cells. 1. mRNA level in non-transfected INR-32 human neuroblastoma cells. 2. mRNA level in INR-32 human neuroblastoma cells transfected by the pCI-neoAPP vector. Data are presented as mean \pm SD of 3 independent experiments performed in duplicate. Statistical significance was determined by *t* test. Differences between APP gene fragment expression are statistically significant ($p < 0.001$).

Results

Expression of opioid receptor in INR-32 human neuroblastoma cells

The expression of opioid receptors by cells subjected to BACE1-opioid peptide siRNA delivery was measured. The expression of MOR, DOR, and KOR mRNA in INR-32 human neuroblastoma cells was studied by using quantitative real-time RT-PCR (Figure 1). All 3 opioid receptors were found to be expressed in the analyzed cells, although the MOR receptor was predominantly expressed and the MOR: KOR: DOR ratio was 4.2:1.5:1.

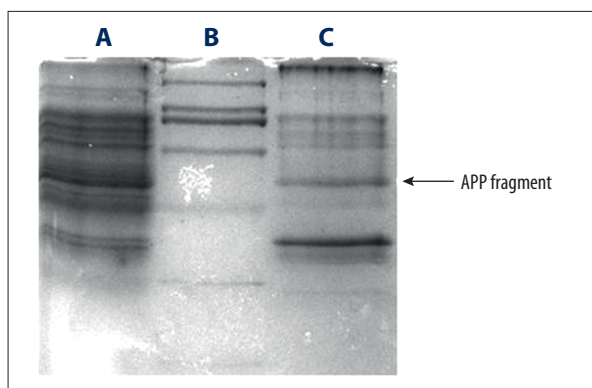


Figure 3. The expression of the APP gene on protein level in INR-32 human neuroblastoma cells. (A) APP protein expression in INR-32 human neuroblastoma cells transfected by the pCI-neoAPP vector. (B) Protein molecular mass standards (97400, 66200, 45500, 31000, 21500, 14400, 6500 kDa). Lane C. APP protein expression in non-transfected INR-32 human neuroblastoma cells. Total cell protein lysates were electrophoresed on 12% polyacrylamide Tris-tricine gels and visualized by Coomassie stain after sonication and centrifugation.

Construction of pCI-neoAPP vector. Transfection and expression level APP in INR-32 human neuroblastoma cells

The present study used a constructed pCI-neoAPP expression vector with the APP gene as a key target for BACE1 expression study in human neuroblastoma cells. The sequenced pCI-neoAPP vector containing the amplified fragment coding the 240 C-terminal amino acids of human APP was used for expression in the INR-32 human neuroblastoma cell line. The cloned APP fragment contains the predicted sites for cleavage by α , β , or γ -secretase. After stable transfection, the expression of APP mRNA and protein by the INR-32 human neuroblastoma cell line increased by 15 to 20 times (Figures 2 and 3).

According to real-time RT-PCR analysis, statistically significant differences were observed non-transfected and transfected cells with regard to mRNA level ($p < 0.05$). Data is presented as mean \pm SD of 3 independent experiments performed in duplicate.

The relationship between APP gene expression and protein level in total cell extract was evaluated in cells transfected by the pCI-neoAPP vector and those which were not. On the protein level, gene expression was found to be few times higher in transfected cells (Figure 3B) than in non-transfected cells (Figure 3A). No difference was seen between transfected and non-transfected cells with regard to morphology or cell kinetics. Western blotting confirmed observed differences in INR-32 human neuroblastoma cell extracts (Figure 4A, 4B). A stable APP-INR-32 human neuroblastoma line was used to measure the expression and activity of the BACE-1 enzyme.

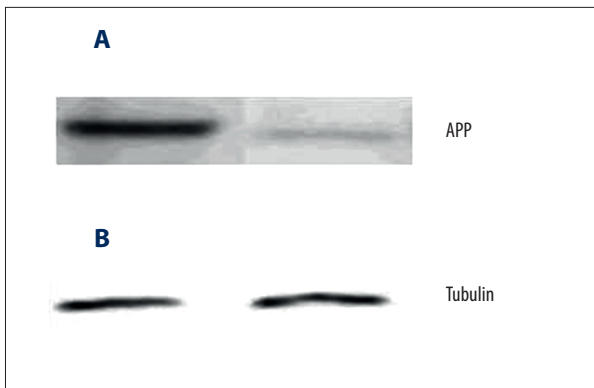


Figure 4. Western blot analysis of APP protein level in INR-32 human neuroblastoma cells. (A) INR-32 human neuroblastoma cells transfected by pCI-neoAPP vector. (B) Non-transfected INR-32 human neuroblastoma cells.

Opioid-siRNA construction. Incubation with APP-INR-32 human neuroblastoma cells

The greatest inhibition of BACE expression (86.5%) was demonstrated by siRNA A5 3'-3'GCAAGGAGUACAACUAUGAUU 5', 3'GGAGGGAGCAUGAUCUUGUU5' and control siRNA 3'UAGCGACUAAACACA UCAAUU 5'. The tested siRNA A5 cross-linked with the opioid used for BACE1 siRNA-opioid peptide for delivery to the cells by receptor-mediated delivery.

Receptor binding assay

The opioid-peptide and opioid-BACE1-siRNA affinities for the MOR receptor were determined in APP-INR-32 human neuroblastoma cells by binding assay. The IC50 values for opioid-peptide and opioid-BACE1-siRNA-1 were 2.03 ± 0.11 and 3.56 ± 0.19 nM, respectively. Of all 5 analogs tested, 2, 3, 4, and 5 showed opioid receptor affinity, with IC50 values of 18.3 ± 1.5 , 14.5 ± 0.92 , 13.9 ± 1.3 and 17.3 ± 1.01 nM, respectively. Obtained results confirmed opioid-BACE1-siRNA-1 forms the strongest bonds with the opioid receptor in human neuroblastoma cells. BACE1-siRNA-1 was used in the following experiments.

The effect of opioid siRNA on BACE1 expression in APP-INR-32 human neuroblastoma cells

Opioid-siRNA transfection

After transfection, BACE1 gene expression on the mRNA and protein levels was measured in APP-INR-32 human neuroblastoma cells with the opioid-1 construct. Obtained results revealed no significant change in BACE-1 gene expression in cells transfected with the opioid-BACE1-siRNA-1 construct on the mRNA level (Figure 5) but by around 3.5 times on the protein level (Figure 6). Western blotting was used to confirm

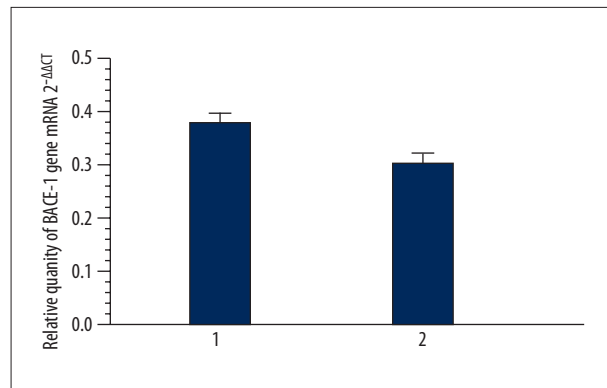


Figure 5. BACE1 gene expression in INR-32-APP human neuroblastoma cells, measured by ELISA 1. INR-32-APP human neuroblastoma cells before transfection of opioid-BACE1-siRNA-1 construct 2. INR-32-APP human neuroblastoma cells after transfection of opioid-BACE1-siRNA-1 construct. Data are presented as mean \pm SD of 3 independent experiments performed in duplicate. Statistical significance was determined by *t* test. Differences between BACE-1 gene expression are statistically significant ($p < 0.001$).

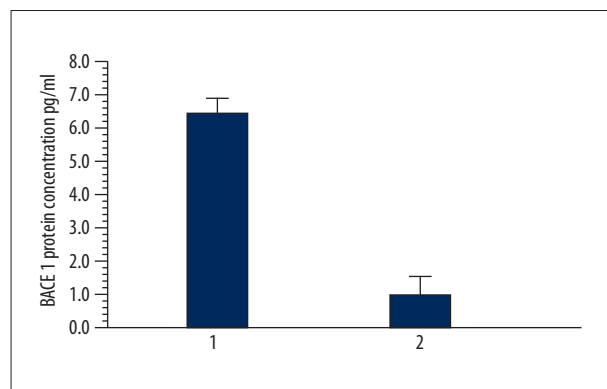


Figure 6. BACE1 protein expression in INR-32-APP human neuroblastoma cells. 1. INR-32-APP human neuroblastoma cells before transfection of opioid-BACE1-siRNA-1 construct. 2. INR-32-APP human neuroblastoma cells after transfection of opioid-BACE1-siRNA-1 construct. Data are presented as mean \pm SD of three independent experiments performed in duplicate. Statistical significance was determined by *t* test. Differences between BACE-1 gene expression on protein level are statistically significant ($p < 0.001$).

observed differences in transfected INR-32 human neuroblastoma cell extracts (Figure 7A, 7B) and confirm that the correct protein was being tested.

BACE immunoblotting showed that the BACE siRNA treatment decreased BACE levels as compared to the control siRNA treatment and the untreated cells. The results show that the treatment of APP-INR-32 cells with BACE siRNA, which reduces the level of BACE mRNA, reduced BACE protein levels, and reduced

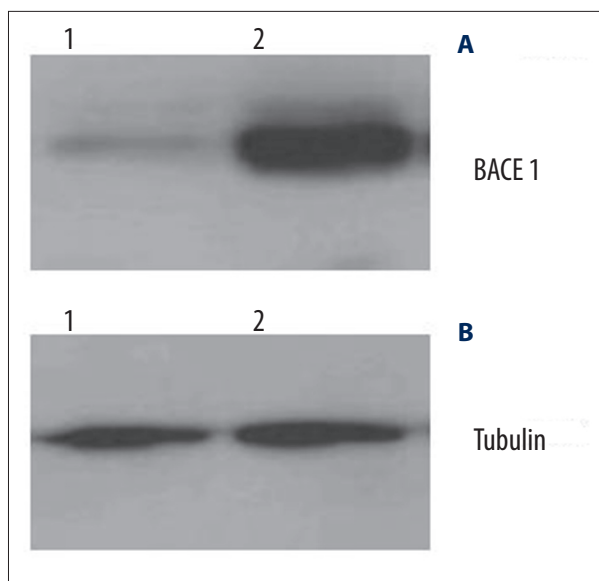


Figure 7. Western blot analysis of BACE 1 protein level in INR-32 human neuroblastoma cells. (A) – BACE-1 protein level, (B) – tubulin protein level. 1. -INR-32 human neuroblastoma cells transfected by opioid-BACE1-siRNA-1 construct. 2. -INR-32 human neuroblastoma cells non transfected by opioid-BACE1-siRNA-1 construct.

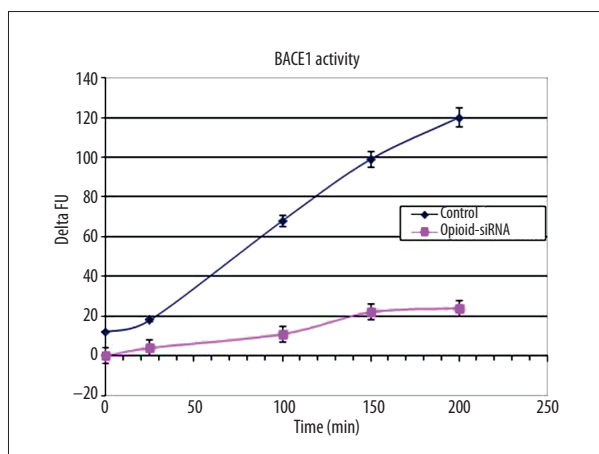


Figure 8. BACE1 activity in INR-32 human neuroblastoma cell extracts. BACE1 activity was assayed using the BACE1 Activity Assay Kit. The results are presented as the Δ Fluorescence Units (Δ FU), ie, the fluorescence detected in the sample with and without the opioid-siRNA construct. The fluorescence was followed for 4 h. The Δ represents the net fluorescence obtained specifically from BACE1 activity.

A β generation by about 20% (data not shown). The cells transfected with the opioid-siRNA construct were found to be identical to the non-transfected cells, or those transfected with control siRNA, with regard to morphology and cell kinetics.

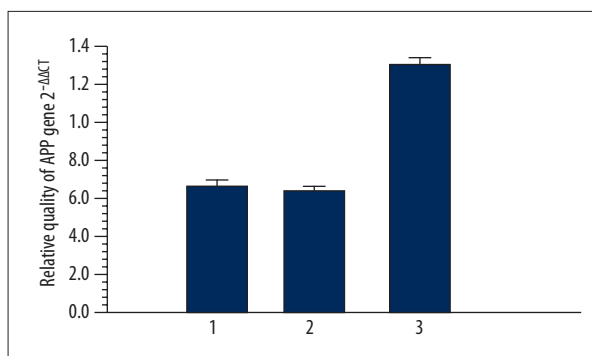


Figure 9. The influence of APP gene expression on mRNA level in INR-32-APP human neuroblastoma cells. 1. INR-32-APP human neuroblastoma cells not transfected with opioid – BACE1-siRNA-1 construct. 2. INR-32-APP human neuroblastoma cells after transfection of opioid –BACE1-siRNA-control construct. 3. INR-32-APP human neuroblastoma cells after transfection of opioid – BACE1-siRNA-1 construct. Data are presented as mean \pm SD of 3 independent experiments performed in duplicate. Statistical significance was determined by *t* test. Differences between analyzed APP gene expression are statistically significant ($p < 0.001$)

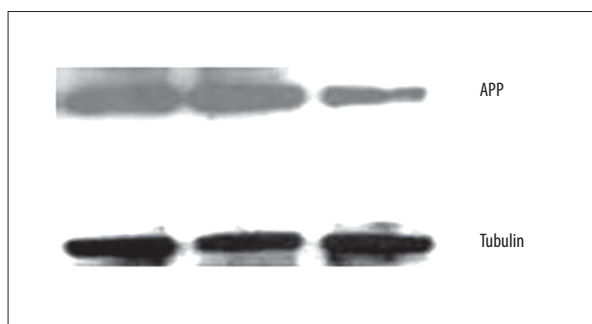


Figure 10. Western blot analysis of BACE 1 protein level in INR-32 human neuroblastoma cells. 1. INR-32-APP human neuroblastoma cells not transfected with opioid-BACE1-siRNA-1 construct. 2. INR-32-APP human neuroblastoma cells after transfection of opioid-BACE1-siRNA-1 control construct. 3. INR-32-APP human neuroblastoma cells after transfection of opioid-BACE1-siRNA-1 construct.

BACE1 activity in INR-32 human neuroblastoma cells

BACE 1 activity was measured in INR-32 human neuroblastoma cell extracts using 10 μ g of total cell protein per reaction using a BACE1 Activity Assay Kit. The activity was measured in the presence or absence of the opioid-siRNA. The difference between the fluorescence obtained by the samples (Δ FU) represents the BACE1 activity. As shown in Figure 8, BACE1 activity was detected in INR-32 human neuroblastoma cells. Internalization of the opioid-BACE-1 siRNA complex to the cells significantly decreased BACE 1 activity by 6 times. APP gene

expression on mRNA and protein level increased by 2 times after transfection of INR-32 – APP cells by the opioid-BACE-siRNA-1 construct (Figures 9 and 10)

Discussion

The rapid pace of recent pharmaceutical drug discovery has resulted in the emergence of increasing numbers of novel therapeutic drugs for the treatment of a variety of diseases. Alzheimer disease (AD) is the main cause of dementia in the elderly. In recent years, the biological processes involved in the pathogenesis of AD have become increasingly better understood. The accumulation of insoluble aggregates of amyloid-beta peptide is thought to be the central mechanism for the pathogenesis of AD, which contributes to memory impairment. Amyloid-beta peptides are generated from the cleavage of amyloid precursor protein (APP) by β - and γ -secretases, and cleavage by BACE1 is believed to be a prerequisite for processing mediated by γ -secretase.

The A β is secreted into the extracellular spaces and accumulates to form aggregates, fibrils, and eventually amyloid deposits called senile plaques. The 2 types of A β formed from secretase cleavage are A β 40 and A β 42, with the latter being the prevalent form found in senile plaques. Blocking the activity of BACE1 or γ -secretase can inhibit the production of A β peptides. As decreases in the levels of BACE and APP could lead to reduction in A β levels, the identification of proteins or compounds that block secretase activity has been, and remains, a major goal of AD research, and intense interest has been generated in studying the inhibition of β and γ -secretases for therapeutic intervention in AD patients [27–29]. Knockout studies show that BACE1 is critical for A β generation; for example, transgenic mice lacking BACE1 do not produce A β , and importantly, show an otherwise normal phenotype with no detrimental effects on viability or morphology [30]. Hence, BACE1 inhibition could be achieved without developing toxic adverse effects. This observation has made BACE1 an important target for drug discovery research.

The *in vitro* introduction of nucleic acids or proteins into cells offers great possibilities in many different branches of biological and biomedical study, and there are many methods of doing this. Transport of macromolecules across the blood-brain barrier (BBB) requires both specific and nonspecific interactions between macromolecules and proteins/receptors expressed on the luminal and/or the abluminal surfaces of the brain capillary endothelial cells. Endocytosis and transcytosis play important roles in the distribution of macromolecules. Transfection of chemically synthesized short interfering RNAs (siRNAs) enables a high level of sequence-specific gene silencing. Although siRNA design algorithms have been improved in recent years, it is still necessary to confirm the functionality of a given siRNA experimentally.

siRNA therapy is a new molecular approach in the search for efficient AD therapy based on the principle of RNA interference. Some studies using RNA interference (RNAi) technology have examined the ability of siRNA to silence BACE. Previous findings have shown that no apparent adverse effects are observed in BACE1-deficient mice [31,32]. Kao et al. reported that BACE1 siRNA specifically influences the β -cleavage of APP and may be a potential therapeutic approach for treating AD. Suppression of BACE1 expression by siRNA was not found to change the subcellular distribution of APP and Presenilin 1, indicating that loss of BACE1 elicits no profound cellular defects [33]. This finding confirms that BACE 1 is a potential therapeutic target for the treatment of AD.

Dong et al. [34] observed that treatment with BACE and APP siRNAs can decrease levels of BACE, full-length APP, and APP c-terminal fragments. In another study, a pAZLDC2 vector was constructed to deliver the siRNA for silencing the BACE 1 gene [35].

The directed delivery of bioactive reagents into cells is one of the most intensely pursued objectives in biomedical research, yet few major breakthroughs have occurred that can be broadly applied in both laboratory research and therapeutic applications. Receptor-mediated delivery is based on simple biological principles and uses existing ligand-induced internalization pathways to deliver a wide variety of biomedically active molecules into the cytoplasm and the nucleus [36,37]. Kossiakoff et al. [33] developed a robust delivery method for the transport of proteins to the cytoplasm of mammalian cells without compromising the integrity of the cell membrane, using a variant of substance P, a neuropeptide that is rapidly internalized upon interaction with the neurokinin-1 receptor (NK1R). Zhang et al. [38] reported a new strategy for cell-type-specific delivery of functional siRNAs into folate receptor-expressing cells. The described method involves the non-covalent attachment of siRNAs to ligand-conjugated oligodeoxynucleotides via nucleic acid base-paired interactions.

The present study uses receptor-mediated delivery to introduce BACE1 siRNA to cells. BACE1 siRNA was conjugated with an opioid-peptide complex, which was then delivered to stable INR-32-APP human neuroblastoma cells. The results demonstrate that the opioid-BACE1-siRNA-1 construct bound to opioid receptors in human neuroblastoma cells and internalized. After BACE1-siRNA-1 internalization, no significant change was noted in BACE-1 mRNA expression, but a 3.5-fold reduction was observed in the protein level. The expression of APP mRNA and protein doubled after transfection of INR-32-APP cells by the opioid-BACE-siRNA-1 construct. Our results show that the construct was delivered to the studied cells bound with the specific opioid receptor, then inhibited BACE gene expression.

Conclusions

The results of this study show that the opioid-BACE-siRNA1 construct used in the study may effectively inhibit the A β produced from amyloid precursor protein (APP) through proteolytic

processing by the aspartyl protease β site APP-cleaving enzyme (BACE). Receptor-mediated delivery of BACE1 siRNA to cells should be considered an important approach in developing AD therapeutics and offers much promise for new treatment strategies in Alzheimer's disease.

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