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Nasal Administration of Cationic Nanoemulsions as *CD73-siRNA* Delivery System for Glioblastoma Treatment: a New Therapeutical Approach

J. H. Azambuja[1] · R. S. Schuh[2] · L. R. Michels[2] · N. E. Gelsleichter[1] · L. R. Beckenkamp[1] · I. C. Iser[1] · G. S. Lenz[1] · F. H. de Oliveira[3] · G. Venturin[4] · S. Greggio[4] · J. C. daCosta[4] · M. R. Wink[1] · J. Sevigny[5,6] · M. A. Stefani[7] · A. M. O. Battastini[8] · H. F. Teixeira[2] · E. Braganhol[1]

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Abstract

Glioblastoma is the most devastating primary brain tumor. Effective therapies are not available, mainly due to high tumor heterogeneity, chemoresistance, and the difficulties imposed by blood-brain barrier. CD73, an enzyme responsible for adenosine (ADO) production, is overexpressed in cancer cells and emerges as a target for glioblastoma treatment. Indeed, ADO causes a variety of tumor-promoting actions, particularly by inducing tumor immune escape, whereas CD73 inhibition impairs tumor progression. Here, a cationic nanoemulsion to deliver CD73siRNA (NE-siRNA CD73R) via nasal route aiming glioblastoma treatment was developed. NE-siRNA CD73R was uptaken by glioma cells in culture, resulting in a parallel 60–80% decrease in AMPase activity and 30–50% in cell viability. Upon nasal delivery, NE-siRNA CD73R was detected in rat brain and serum. Notably, treatment with CD73siRNA complexes of glioma-bearing Wistar rats reduced tumor growth by 60%. Additionally, NE-siRNA CD73R treatment decreased 95% ADO levels in liquor and tumor CD73 expression, confirming in vivo CD73 silencing. Finally, no toxicity was observed in either primary astrocytes or rats with this cationic nanoemulsion. These results suggest that nasal administration of cationic NE as CD73 siRNA delivery system represents a novel potential treatment for glioblastoma.

Keywords Adenosine · Brain delivery · Cationic nanoemulsion · CD73 · Glioma

Highlights
- Nasal administration of nanoemulsions was efficient in CD73siRNA rat brain delivery.
- NE-siRNA CD73 was uptaken by tumor cells, resulting in in vitro and in vivo CD73 knockdown.
- NE-siRNA CD73 treatment impaired tumor growth in a preclinical glioblastoma model.
- Cationic nanoemulsions did not induce in vitro and in vivo toxicity.
- NE-siRNA CD73 may represent a new therapeutic approach for glioblastoma treatment.

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✉ J. H. Azambuja
julianahazambuja@hotmail.com

[1] Programa de Pós-Graduação em Biociências, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

[2] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[3] Departamento de Patologia, UFRGS, Porto Alegre, RS, Brazil

[4] Brain Institute of Rio Grande do Sul (BraIns), Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil

[5] Département de Microbiologie-Infectiologie et d'Immunologie, Faculté de Médecine, Université Laval, QC, Québec, Canada

[6] Centre de recherche du CHU de Québec, Université Laval, Québec City, QC G1V 4G2, Canada

[7] Departamento de Morfologia, UFRGS, Porto Alegre, RS, Brazil

[8] Departamento de Bioquímica, UFRGS, Porto Alegre, RS, Brazil

Abbreviations

ADO	adenosine
ATCC	American Type Culture Collections
BBB	Blood-brain barrier
CD73	Ecto-5′-nucleotidase
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAPI	4′, 6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DOTAP	1,2-dioleoyl-sn-glycero-3-trimethylammonium propane
FBS	Fetal bovine serum
HPCL	High-performance liquid chromatography
INO	Inosine
NE	Nanoemulsion
NE-siRNA CD73R	nanoemulsion loaded rat CD73 siRNA
NE-siRNA CD73H	nanoemulsion loaded human CD73 siRNA
NE-siRNA scramble	nanoemulsion loaded GFP siRNA
NR-NE	Nile red label nanoemulsion
MCT	Medium chain triglycerides
PCS	Photon correlation spectroscopy
siRNA	Small interfering ribonucleic acids
RT	Room temperature
TCA	Trichloroacetic acid
TMZ	Temozolomide

Introduction

Cancers of the Central Nervous System (CNS) are extremely complex and heterogeneous. Glioblastoma, also called grade IV glioma, is the most lethal and aggressive CNS tumor [1]. The standard therapy includes surgery, followed by chemotherapy and radiotherapy [2]. Furthermore, a combination of the infiltrative capacity of glioblastoma cells, their high proliferative rate, the development of chemoresistance, increased angiogenesis, and the structural complexity of the brain, results in high tumor recurrence rates and in poor response to chemotherapy [3, 4]. In consequence, the disease ultimately follows a fatal course with a median survival of 12 to 15 months, and only 10% of patients survive more than 5 years [5, 6]. In addition, delivery of therapeutic molecules into the CNS remains the major clinical challenge that has not yet been surmounted. To overcome difficulties in glioblastoma therapy, novel strategies such as molecular targeted therapy, gene therapy, and nanotechnology have emerged from the interface between preclinical and clinical research [7–9].

In a previous study, our group showed CD73 as a promising target for glioblastoma treatment [10]. In addition to producing adenosine (ADO) from AMP hydrolysis, which is related to tumor growth, angiogenesis, metastasis, escape from immune surveillance, and chemoresistance [11–17], CD73 exhibits cell-cell and cell-extracellular matrix adhesion properties, being involved in cell migration and invasion processes [10, 18, 19]. On the other hand, the inhibition of its expression and enzyme activity leads to better prognosis and decreased tumor growth in preclinical models of glioblastoma and breast cancer [10, 20–23].

The use of small interfering ribonucleic acid (siRNA) molecules represents a promising tool for gene expression inhibition, constituting an interesting therapeutic strategy for cancer treatment, including brain tumors [24, 25]. siRNA is a mechanism exerted from a RNA double strand of approximately 19–23 nucleotides that triggers the cleavage of specific sequences of mRNA, resulting in inhibition of gene expression either at the translation phase, or hindering the transcription of specific genes [26–28]. For antitumor therapy, siRNA sequences have typically been administered locally, via intracerebral/intratumoral injections, or systemically, intravenously, using liposomal systems as nucleic acid carriers, providing an excellent therapeutic opportunity for brain tumors [24, 29]. However, in order to translate siRNA from an experimental approach to a viable therapeutic strategy for brain tumors, there is a critical need for a safe and effective delivery system, allowing the therapeutic effect to be obtained with no damage to healthy cells.

The nasal route has recently been considered as an efficient and non-invasive way of administering molecules and drugs to the CNS [30–33]. This advantage is related to the anatomical link of the nasal cavity to the CNS via olfactory pathway, which overcomes the blood-brain barrier (BBB) [34]. However, the nasal epithelium route has some limitations, such as poor absorption and the presence of a mucus layer and its continuous clearance from nasal mucosa [35, 36]. For this reason, it is necessary to develop a safe and effective system that could be administered through the nasal route to improve the intracellular siRNA delivery to the brain. Cationic nanoemulsions (NE) have been emerging as promising systems for brain targeting, as these nanostructures efficiently interact with negatively charged siRNAs, forming complexes that allow cell uptake and consequent interaction with the intracellular target of siRNA sequences [37, 38]. Moreover, these systems are an attractive alternative due to their ability of protecting nucleic acids from metabolism and/or chemical degradation, increasing half-life in the bloodstream, and tissue biodistribution [37–40]. Therefore, the aim of this study was to develop and to evaluate the therapeutic efficacy of NE as CD73siRNA delivery system administered through nasal route for glioblastoma treatment.

Materials and Methods

Preparation and Characterization of siRNA/Nanoemulsion Complexes

Cationic nanoemulsions (NE) were prepared by microfluidization as described by Schuch and colleagues [40], and complexes comprised their association with siRNA duplex by adsorption at different charge ratios. Blank NE was composed by lecithin (Lipoid, Ludwigshafen, GER), medium chain triglycerides (MCT, Lipoid, Ludwigshafen, GER), 1,2-dioleoyl-sn-glycero-3-trimethylammonium propane (DOTAP) (Lipoid, Ludwigshafen, GER), and ultrapure water. Fluorescent formulations were prepared as described above by adding Nile Red fluorescent stain (NR) (0.1 mg/mL). These formulations were labeled as NR-NE.

Complexes were prepared by the adsorption of siRNA duplex to blank formulations. The adsorption was performed by the addition of previously established amounts of siRNA to NE at RT, resulting in NE-scramble siRNA (GFPsiRNA), NE-siRNA CD73R (rat CD73siRNA), and NE-siRNA CD73H (human CD73siRNA) complexes. All the complexes were prepared at the theoretical + 0.1/− 1, + 1/− 1, + 2/− 1, and + 4/− 1 charge ratios (ratios of the positive charges from cationic lipids to the negative charges from siRNA phosphate groups).

Qualitative assessment of siRNA complexation efficiency was also performed using an agarose gel retardation assay based on the principle that when siRNA is efficiently bound to DOTAP in the NE interface, siRNA migration through the gel will be completely retarded. Complexation of the siRNA sequences with the cationic formulations was verified by agarose gel electrophoresis. The complexes were electrophoresed for 15 min in 1% agarose gel stained with SYBR®Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, USA). Naked siRNA was used as control. Films obtained in agarose gel electrophoresis procedure were scanned to capture their images. Optical density (OD) of each gel was measured using the NIH ImageJ 1.44b software (https://imagej.nih.gov/ij/).

Mean droplet size, polydispersity index, and ζ-potential of the formulations and complexes were determined by photon correlation spectroscopy (PCS) at 90° and electrophoretic mobility measurements (3000HS Zetasizer, Malvern Instruments, Worcestershire, UK). The samples were diluted in water.

Assessment of Cellular Uptake of NE-siRNA Complexes

C6 glioma cell line was used to evaluate cellular uptake of NE-siRNA complexes. Cells were purchased from American Type Culture Collections (ATCC, Rockville, USA), cultured in Dulbecco's Modified Eagle Medium (DMEM, pH 7.4) supplemented with 10% fetal bovine serum (FBS) maintained at 37 °C under 5% CO_2 atmosphere.

To evaluate their cellular uptake and internalization, NR-NE-siRNA fluorescent formulations were used. Briefly, 2×10^4 cells were seeded in 48-well plates; the transfection mix (1 μM of siRNA complexed with NE at + 2/− 1 and + 4/− 1 charge ratios) was prepared and added to cell cultures. After 48 h of incubation, cells were washed with cold PBS (pH 7.4) and stained with fluorescent DNA-binding DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) (1:10,000) for 15 min and Alexa Fluor®488 Phalloidin (Thermofisher, Waltham, MA) for 30 min to stain nucleus and cytoskeleton, respectively. Digital images were captured in IN CELL Analyzer 2200 (GE Healthcare Life Sciences, Marlborough, USA).

Determination of AMPase Activity in Cell Cultures

To evaluate in vitro CD73 silencing, C6 glioma cells and Hacat human non-transformed cells were exposed to NE-siRNA complexes for 48 h as described above. AMPase activity was analyzed as described by Azambuja and colleagues [41, 42]. The released inorganic phosphate (Pi) was assayed by malachite green method and KH_2PO_4 as standard [43]. Total protein content was measured by the Coomassie blue method according to Bradford [44] using serum albumin as standard. Activity was reported as nmol Pi/min/mg of protein.

Cytotoxicity Evaluations

The cytotoxic potential of NE-siRNA CD73R was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, USA) assay in a glioblastoma cell line (C6) and in a rat astrocyte primary cultures, which was used as a healthy cell model. Primary cortical astrocyte cultures were prepared as described by Pacheco and colleagues [45] (Approval protocol number 293/14). Confluent astrocyte cultures or C6 cells were exposed to NE-siRNA (scramble siRNA or siRNA CD73R) as described above ("Assessment of cellular uptake of NE-siRNA complexes" section). Following 48 h of treatment, cell viability was assessed by MTT according to the manufacturer's instructions. Absorbance was determined at 492 nm using a Spectramax microplate reader (Molecular Devices, Sunnyvale, USA). The cell viability percentage was calculated from the absorbance values relative to those of untreated cells.

In Vivo Studies

Biodistribution of Fluorescent NE-siRNA CD73R Complexes in Rats

siRNA duplexes were adsorbed in Nile Red-labeled cationic nanoemulsions (NR-NE-siRNA CD73R), as described above, at + 4/− 1 charge ratio. Animals were anesthetized using a

mixture of isoflurane and oxygen (3%–4% induction and 2%–3% maintenance) and randomly divided in two groups: the first group received a nasal administration of PBS, while the second group received NR-NE-siRNA CD73R (10 μg siRNA/Kg). Rats were euthanized at 6, 18, 24, and 32 h following administration.

Dosing was performed by the administration of 32 μL of either PBS or complexes in each nostril using a micropipette (Eppendorf P-200). Blood and brain samples were collected. Serum was separated from whole blood after centrifuging at 2000 ×*g* for 20 min at 4 °C. Brain and serum ex vivo fluorescence was determined using an MS FX PRO (Bruker Corporation, Billerica, USA), and fluorescence measurements in average radiant efficiency were determined using Molecular Imaging software (Bruker Corporation). Fluorescence intensity of PBS-treated rats was subtracted to correct for fluorescence background. The results were scanned with both 541 and 488 nm excitation. Data were expressed as MFI.

Glioma Preclinical Model and In Vivo CD73 Gene Silencing

C6 glioma cells were implanted in male Wistar rats (250–300 g, 8 weeks old) as described by Azambuja et al [10]. Five days after glioma implantation, animals were randomly divided into three groups as follows ($n = 7$): (1) Control (PBS-treated); (2) NE-siRNA scramble (GFP siRNA/nanoemulsion complexes at + 4/− 1 charge ratio); and (3) NE-siRNA CD73R (CD73 siRNA/nanoemulsion complexes at + 4/− 1 charge ratio). The formulations were administered nasally, 12 h/12 h, at doses of 10 μg/kg, for 15 days. At the end of the protocol (20 days following glioma implantation), rats were euthanized and brains were removed, sectioned, and frozen. At least three Hematoxylin and Eosin (HE) sections (5 μm frozen tissue) from each animal were analyzed by a pathologist. For tumor size quantification, images were captured using a digital camera connected to a microscope (Olympus BX-51, Tokyo, Japan) and the tumor area (mm^2) was determined using ImageJ software. The total volume (mm^3) of the tumor was computed by the multiplication of the slice sections and by the sum of the segmented areas.

For immunohistochemical analysis (IHC), cryostat sections (5 μm) were fixed in a solution containing 95% acetone plus 5% formalin, blocked in 1% albumin solution, and incubated overnight (4 °C) with the following specific antibodies: mouse anti-rat CD31/PECAM-1 mAb (1:30, 550300, BD Pharmingen, BD Biosciences, Mountain View, USA) and rabbit anti-rat CD73 (1:2000; http://ectonucleotidases-ab.com). Thereafter, tissue sections were incubated with the anti-mouse Alexafluor 594 (1:1000, A-21236, Molecular Probes) and anti-rabbit Alexafluor 488 Ab (1:500, A-11008, Molecular Probes Oregon, USA) secondary antibodies and stained with fluorescent DNA-binding DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) (1:10,000) for 15 min. All immunohistological evaluations were done in randomly chosen fields (200×) per tumor (Olympus BX-51, Tokyo, Japan).

All procedures used in the present study followed the Principles of Laboratory Animal Care from NIH and were approved by the Ethical Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (protocol number 293/14) [46].

Analysis of Purine Levels in Cerebrospinal Fluid (CSF)

CSF was drawn (80 μL per rat) by direct puncture of cisterna magna using a syringe (27 gage × 1/2 in length) in Wistar rats anesthetized with ketamine and xylazine at the 20th day after glioma implant. Samples were centrifuged and stored at − 80 °C for further analysis. ATP, ADP, AMP, adenosine, inosine ,and hypoxanthine were analyzed by high-performance liquid chromatography (HPLC) according to Azambuja and colleagues [10], in a reversed-phase HPLC Shimadzu Prominence (Shimadzu, Kyoto, Japan) using a Shimadzu column Shim-pack CLC (M) C18 (150 × 4.6 mm × 5 μm) attached to a guard column Shimadzu Shim-pack GVP-ODS (4.6 × 10 mm). Elution was carried out applying a linear gradient from 100% solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium chloride, pH 5.0) to 100% of solvent B (solvent A plus 30% methanol) over a 42-min period (flow rate at 1.0 mL/min). Purine levels were measured by absorption at 254 nm. The retention time of standards was used as parameter for identification and quantification by comparison of the peak area. Purine levels were expressed as μM [10].

Tolerability Studies

Body Weight, Histological, and Biochemical Determinations

Periodic measurements of body weight were made through the course of 15 days of treatment in the three analyzed groups (control, NE-siRNA scramble, NE-siRNA CD73R) using a balance. The results were plotted as percentage of change in body weight as a function of days for all experimental groups. Histological analysis of lung tissue was performed at the end of treatment of glioma-bearing rats in histological tissue sections stained with H&E. A pathologist analyzed the slides in a blinded manner. To assess hepatic and renal function, blood samples were collected at the end of treatment from all experimental groups, and alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and urea were evaluated as described by the manufacturer. Data were expressed as U/L or mg/dL. For hemogram, blood samples were collected using anticoagulant, and hematological parameters (erythrocytes, leukocytes, platelets, hemoglobin) were determined by a

hematology analyzer (ABX Micros 60, HORIBA, Coraopolis, USA).

Behavioral Analysis After 20 days of tumor implantation, animals were exposed to an Open Field apparatus. Open field exploration was carried on a 40 × 45 cm arena, surrounded by 45 cm high walls, made of brown plywood with a frontal glass wall. The floor of the arena was divided into nine equal squares by black lines. Rats were put in the apparatus, placed on its left rear quadrant, and left to freely explore the arena for 5 min. Crossings of the black lines were counted. The number of crossings was used to measure locomotor activity and exploratory behavior [46].

To detect possible impairments in olfactory capacity of the animals due to the treatment, we chose a chocolate candy ball as a food stimulus. Access to food was removed 2 h prior to the experiment. The animals were placed in a conventional rat cage filled with shavings. Food item was buried in a randomly chosen location in the cage. The test ended as soon as the animals held the item or could not find it in 5 min. Additional details about this experiment are found in Supplementary Fig. 1.

Statistical Analysis

Statistics were performed using software GraphPad Prism 5 (Prism GraphPad Software, San Diego, USA) and data were expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test (for multiple comparisons) or Student's t test were performed as appropriate. Differences were considered significant for a p value of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, respectively.

Results

Developed Cationic Nanoemulsions Exhibit Expected Physicochemical Characteristics and Efficiently Complex to siRNA Sequences

After preparation, NE obtained by microfluidization had milky appearance, a normal characteristic of colloidal systems containing oil in the formulation. The physicochemical properties of NE, NE-siRNA scramble, NE-siRNA CD73R, and NE-siRNA CD73H are described in Table 1. All formulations exhibited nanometric size, although it was observed that mean droplet size of NE tended to increase with the addition of siRNA to the blank nanoemulsion (262.7–601.9 nm), as the charge ratio became less positive. The polydispersity index showed the same tendency, suggesting a destabilization of the system in smaller charge ratios, and values were near 0.2 only for NE and + 4/− 1 complexes, indicating a homogeneous distribution of these systems.

The NE blank formulation showed a positive ζ-potential of approximately + 32 mV, which significantly decreased when siRNA was increasingly added to the formulations (NE-siRNA scramble, NE-siRNA CD73R, and NE-siRNA CD73H), suggesting their localization at the emulsion interface. In addition, NE blank formulation exhibited pH of 6.9, which is compatible with nasal mucosa delivery.

The migration of siRNA from siRNA complexes produced at + 0.1/− 1, + 1/− 1, + 2/− 1, and + 4/−1 charge ratios is shown in Fig. 1. Only siRNAs from complexes at + 4/− 1 charge ratio were retained at the application site. The complexation rate of 80–100% for all of the complexes at + 2/− 1 and + 4/− 1 charge ratio was estimated using the software ImageJ (data not shown). Therefore, considering the efficiency of complexation of oligonucleotide sequences, these charge ratios (+ 2/− 1 and + 4/− 1) were applied in the subsequent experiments.

NE-siRNA CD73 Complexes Efficiently Decrease CD73 Activity and Cell Viability in C6 Glioma Cells and Do Not Induce Astrocyte Toxicity

Fluorescence microscopy was employed to visualize the qualitative intracellular uptake of Nile Red-labeled cationic NE (NR-NE-siRNA) complexes in C6 glioma cells following 48 h of incubation. As shown in Fig. 2 (panel a), NR-NE-siRNA complexes were effectively internalized by glioma cells at the tested charge ratios (+ 2/− 1 and + 4/− 1). Additionally, phalloidin cytoskeleton staining was not affected by NR-NE-siRNA treatment; C6 cell showing a normal morphology.

Parallelly, the potential of NE-siRNA CD73R to knockdown CD73 activity in C6 glioma cells was determined. As shown in Fig. 2 (panel b), AMPase activity was decreased by ~ 60% (241.04 nmol Pi/min/mg of protein) and ~ 80% (130.14 nmol Pi/min/mg of protein) following exposure to NE-siRNA CD73R at + 2/− 1 and + 4/− 1 charge ratios, respectively, when compared to control (561.03 nmol Pi/min/mg of protein). Notably, these results were comparable to CD73 silencing when a commercial transfection reagent was applied [10]. Finally, the potential cytotoxicity of the formulations was evaluated in rat primary astrocyte cultures, which was used as a model of untransformed cells from CNS and in a glioblastoma cell line. Cells were exposed to NE-siRNA scramble or NE-siRNA CD73R at + 2/− 1 and + 4/− 1 charge ratios, and cell viability was determined after 48 h by MTT assay. As shown in Fig. 2 (panel c), NE-siRNA scramble did not induce any alteration in C6 glioblastoma cell viability when compared to untreated control. However, C6 cell viability was decreased by ~ 30% and ~ 50% following exposure to NE-siRNA CD73R at + 2/− 1 and + 4/− 1 charge ratios, respectively, when compared to controls suggesting that the viability of the glioma cells is directly affected by the effect of the CD73 NE-siRNA interfering in the protein synthesis of CD73.

Table 1 Physicochemical properties of NE and NE loaded siRNA formulations at +0.1/− 1, + 1/− 1, + 2/− 1, and + 4/− 1 charge ratios

Formulation	Droplet size (nm)	P.I.	Z-potential (mV)
NE	178.2 ± 12.5	0.16 ± 0.08	+ 32.1 ± 3.7
NE-siRNA scramble + 0.1/ −1	527.3 ± 33.2[a]	0.57 ± 0.18	− 23.6 ± 4.1[b]
NE-siRNA scramble + 1/− 1	493.9 ± 39.7[a]	0.50 ± 0.11	− 13.5 ± 3.2[b]
NE-siRNA scramble + 2/− 1	392.5 ± 23.2[a]	0.45 ± 0.10	− 1.2 ± 5.1[b]
NE-siRNA scramble + 4/− 1	289.3 ± 13.2[a]	0.25 ± 0.10	+ 4.8 ± 4.3[b]
NE-siRNA CD73R + 0.1/− 1	581.7 ± 29.3[a]	0.55 ± 0.12	− 31.4 ± 5.9[b]
NE-siRNA CD73R + 1/− 1	507.4 ± 31.0[a]	0.52 ± 0.12	− 18.9 ± 3.4[b]
NE-siRNA CD73R + 2/− 1	389.0 ± 22.9[a]	0.48 ± 0.10	− 6.8 ± 3.2[b]
NE-siRNA CD73R + 4/− 1	262.7 ± 12.8[a]	0.23 ± 0.11	+ 3.5 ± 3.0[b]
NE-siRNA CD73H + 0.1/− 1	601.9 ± 28.6[a]	0.55 ± 0.19	− 26.5 ± 5.9[b]
NE-siRNA CD73H + 1/− 1	510.0 ± 54.1[a]	0.42 ± 0.14	− 20.7 ± 4.1[b]
NE-siRNA CD73H + 2/− 1	392.7 ± 19.1[a]	0.38 ± 0.09	− 2.9 ± 1.2[b]
NE-siRNA CD73H + 4/− 1	273.9 ± 13.6[a]	0.19 ± 0.08	+ 0.5 ± 2.7[b]

Results represent the mean ± standard deviation of three experiments; difference before and after complexation. Student's *t* test, $p < 0.05$

[a] Mean diameter

[b] ζ-potential

Interestingly, the graph of AMPase enzyme activity (Fig. 2, panel b) exhibits a profile very similar to the graph of glioma cell viability (Fig. 2, panel c). In addition, the complexes did not induce any alteration in astrocyte viability (Fig. 2, panel d) or alteration in AMPase activity on non-transformed Hacat cells when compared to control (Supplementary Fig. 2). Of note, C6 glioma cells show a 30-fold increase in AMPase activity when compared to healthy cells (561.03 and 14 nmol Pi/min/mg of protein for C6 and Hacat, respectively) (Fig. 2b and Supplementary Fig. 2). Therefore, cationic lipid NE complexed to CD73siRNA was efficiently internalized, which was followed by a parallel decrease of AMPase activity and cell viability in glioma cells. Additionally, the formulations were safe to normal cell cultures.

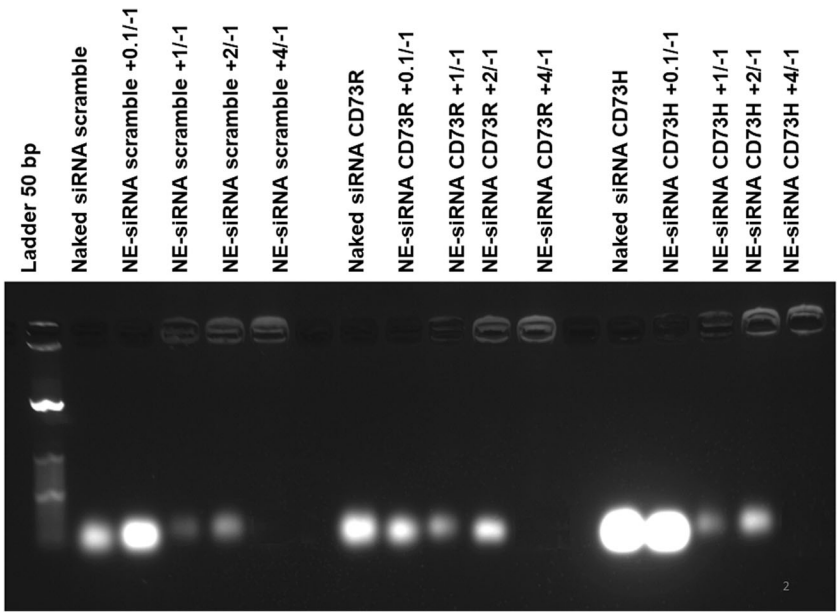

Fig. 1 Migration pattern of siRNA sequences complexed with cationic nanoemulsions. siRNA scramble (GFP), siRNA CD73R, and siRNA CD73H were complexed with cationic NE at charge ratios + 0.1/− 1, + 1/− 1, + 2/− 1, and + 4/− 1. Naked siRNA sequences were applied as free-migration control. Naked or siRNA-NE complexes were allowed to migrate for 15 min on 1% agarose gel. Oligonucleotide bands were stained with SYBR®Gold for visualization. Naked siRNA = free siRNA; NE-siRNA scramble = nanoemulsion complexed with GFP siRNA; NE-siRNA CD73R = nanoemulsion complexed with rat CD73 siRNA; NE-siRNA CD73H = nanoemulsion complexed with human CD73 siRNA

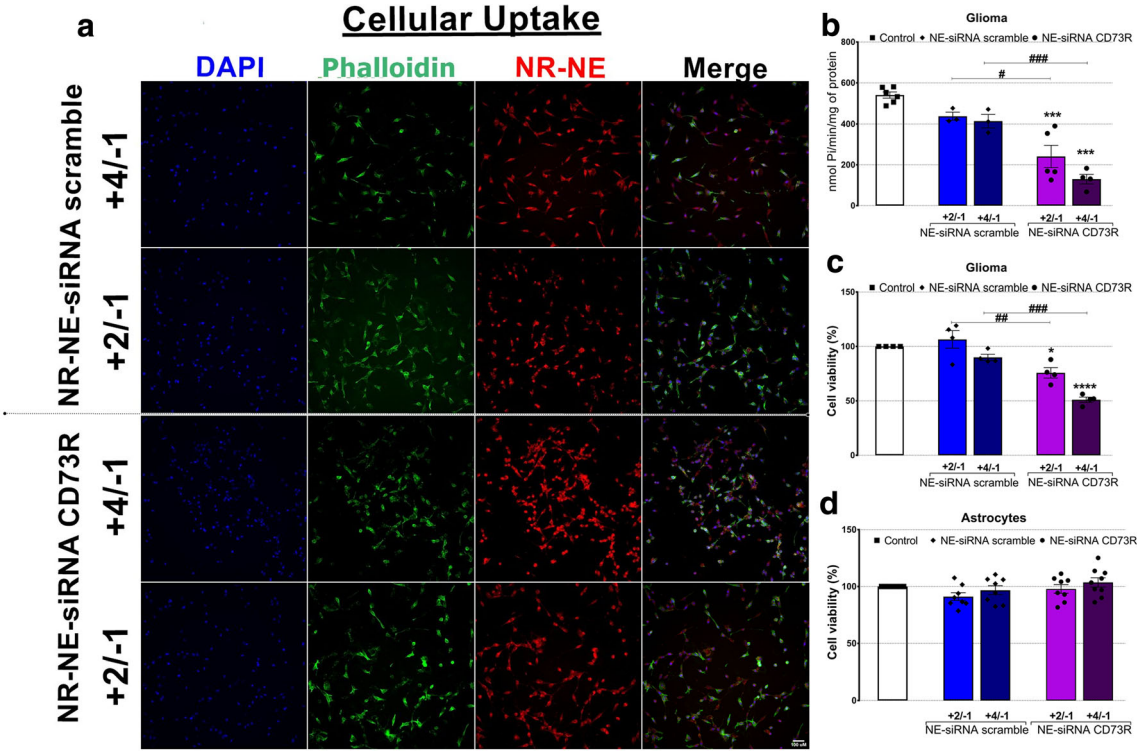

Fig. 2 NE-siRNA CD73R complexes are uptaken by C6 glioma cells and decrease AMPase activity and cell viability. C6 glioma cells were exposed to NE-siRNA scramble or NE-siRNA CD73R at charge ratios + 2/− 1 and + 4/− 1 for 48 h, as indicated. **a** NR-NE-siRNA complexes uptake by C6 glioma cells. Fluorescence microscopy images showing the blue (nucleus-DAPI), red (NR-NE), green (phalloidin-cytoskeleton), and overlay images for NR-NE-siRNA scramble and NR-NE-siRNA CD73R. The images were taken at 10× original magnification. **b** Analysis of AMPase activity in C6 glioma cells exposed to complexes as described above. Data represent mean ± SD of at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by Tukey post hoc. ***Significantly different from control cells (white bar); #, ###significantly different from NE-siRNA scramble ($p < 0.05$ and < 0.001, respectively). **c** Cationic NE-siRNA CD73R exhibit cytotoxicity in a glioblastoma cells line. C6 were exposed to NE complexes as described above and cell viability was determined by MTT assay *, ***Significantly different from control cells (white bar); ##, ###significantly different from NE-siRNA scramble ($p < 0.05$, 0.01 and < 0.001, respectively). **d** Cationic NE does not exhibit cytotoxicity in astrocyte primary cultures. Astrocytes were exposed to NE complexes as described above and cell viability was determined by MTT assay. Cell viability of untreated cells was considered 100%. Values represent the mean ± SD from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by Tukey post hoc for comparisons. NE-siRNA scramble = nanoemulsion complexed with GFP siRNA; NE-siRNA CD73R = nanoemulsion complexed with rat CD73 siRNA

NE-siRNA Complexes Reach CNS Following Nasal Delivery

Considering that NE-siRNA CD73R was uptaken by glioma cells in culture, we further evaluated whether the complexes could reach rat brain and blood serum after nasal delivery. Fluorescent NR-NE-siRNA CD73 at + 4/− 1 charge ratio was chosen for this experiment, since 100% complexation efficiency (Fig. 1) and higher AMPase activity inhibition (Fig. 2b) were observed at this condition when compared to controls. The complexes (10 μg/Kg siRNA) were delivered via nasal route and the fluorescence was determined in rat brain and blood serum following 6, 18, 24, and 32 h of administration, as described in material and methods. Notably, as shown in Fig. 3 (panel a), a fluorescence peak was already detected in rat brain following 6 h of a single nasal administration. The signal intensity increased up to 18 h, which was followed by a gradual decrease, becoming undetectable 32 h after delivery. A fluorescence peak was also detected in blood serum 18 h after complex administration, which gradually decreased up to 24 h, remaining stable within 32 h of delivery, when the formulation was still detectable in the circulation (Fig. 3b). Therefore, data indicated that the developed cationic NE was suitable for CD73siRNA brain delivery via nasal route and suggested a treatment regimen twice a day (every 12 h) in the preclinical glioblastoma model.

NE-siRNA CD73R Treatment Impairs In Vivo Glioblastoma Growth

Since NE-siRNA CD73R was detected in CNS after nasal delivery, the antiglioma effect of the developed formulation was further analyzed in a rat preclinical glioblastoma model. Following 5 days of glioma implant in rat brain, animals were treated with NE-siRNA CD73R for additional 15 days

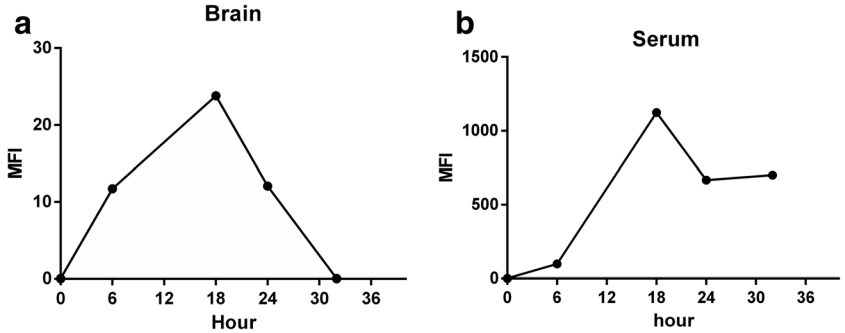

Fig. 3 NR-NE-siRNA CD73R complexes are detected in rat brain and blood serum following nasal administration. NR-NE siRNA CD73R complexes at + 4/− 1 charge ratio were delivered once via nasal route and the fluorescence signal was determined after 6, 18, 24 and 32 h in **a** brain and **b** blood serum using IVES. The values represent the mean (N = 3) NE-siRNA CD73R = nanoemulsion complexed with rat CD73 siRNA

(10 μg/kg; 12/12 h; Fig. 4a). Untreated and NE-siRNA scramble-treated animals were considered controls. At the 20th day of protocol, animals were euthanized and tumors were measured as described in material and methods. The treatment with NE-siRNA CD73R reduced tumor volume by 60% (58.8 ± 32.7 mm^3) when compared to control groups (139.8 ± 33.5 mm^3 and 139.7 ± 35.4 mm^3 for untreated and NE-siRNA scramble, respectively) (Fig. 4b, c). These results are superior to those found by our group using commercial reagent for CD73 siRNA delivery via intracerebroventricular injection, which decreased 45% of glioblastoma growth [10]. On the other hand, no histopathological differences were observed in implanted tumors among the groups (Fig. 4d and Table 2).

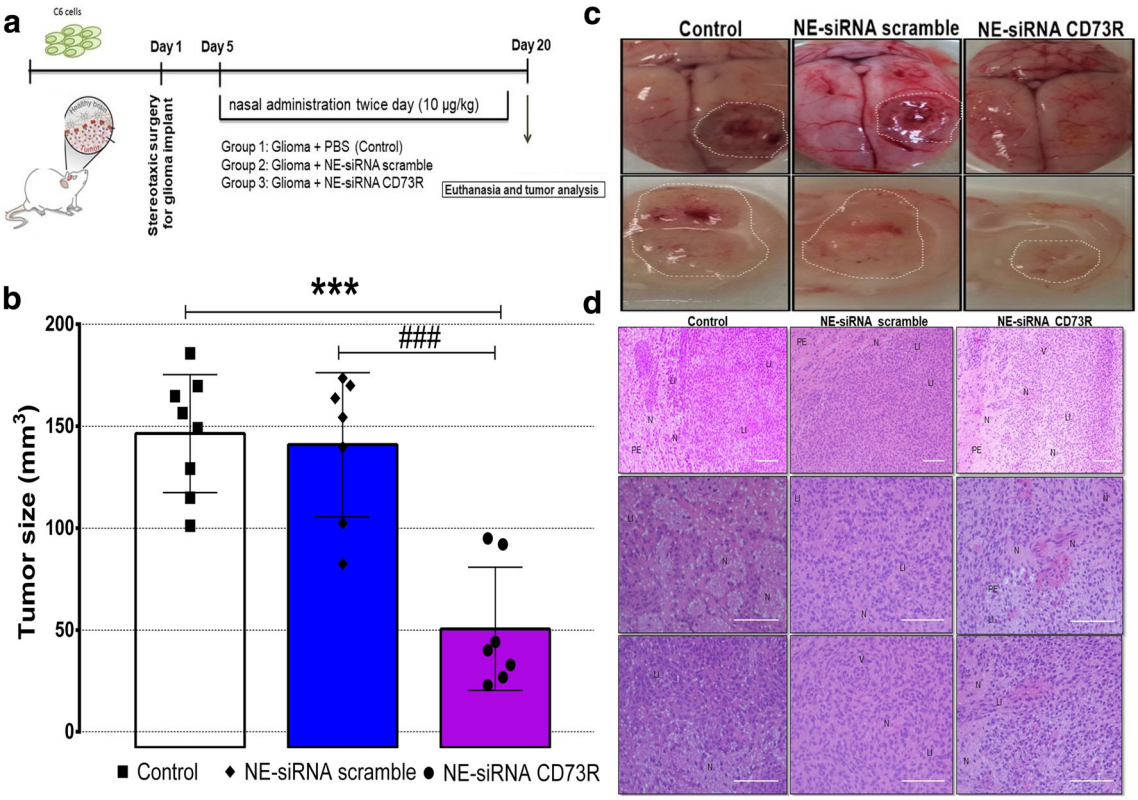

Fig. 4 NE-siRNA CD73R treatment decreases tumor volume in a preclinical glioblastoma model. **a** Timeline of the experiment and the therapeutic scheme employed; **b** Quantification of tumor size in glioma-implanted rats after 15 days of treatment with NE-siRNA complexes. For quantification of tumor size, the images were captured from HE slices using a digital camera connected to a microscope, and the total volume (mm^3) was determined using the Image Tool Software™. Values represent the mean ± SD of at least seven animals per group. Data were analyzed by ANOVA followed by post-hoc comparisons (Tukey's test). ***, ###Significantly different from control (PBS animals) and NE-siRNA scramble treated group, respectively ($p < 0.001$). **c** Representative pictures of tumors; and **d** representative HE of implanted tumors demonstrating histopathological characteristics (PE = peritumoral edema; LI = lymphocyte infiltration; N = necrosis; V = vascular proliferation). The complete analysis is shown in Table 2. Images were taken at 200× magnification

Table 2 Histological characteristics in control or NE-siRNA complexes treated glioma-bearing rats

	Control	NE-siRNA scramble	NE-siRNA CD73R
Coagulative necrosis	3/8	6/7	4/7
Intratumoral hemorrhage	3/8	4/7	3/7
Lymphocytic infiltration	2/8	3/7	3/7
Edema	8/8	7/7	7/7
Vascular proliferation	8/8	7/7	6/7

Five days after glioma implantation, the animals were divided in three groups and treated for 15 days as follow: Control (PBS-treated); NE-siRNA scramble (siRNA scramble GFP); NE-siRNA CD73R (rat siRNA CD73). The histological variables (coagulative necrosis, intratumoral hemorrhage, lymphocytic infiltration, peritumoral edema and vascular proliferation) were regarded as present or absent. Data are analyzed by ANOVA followed by post-hoc comparisons (Tukey's test)

NE-siRNA scramble: nanoemulsion complexed to GFP siRNA; NE-siRNA CD73R: nanoemulsion complexed to rat

To evaluate whether the antiglioma effect of NE-siRNA CD73R was related to in vivo CD73 knockdown and decreased ADO availability in the CNS, CD73 expression and nucleotide/nucleoside levels were determined by IHC and HPLC analyses, respectively. First, it is interesting to note the higher CD73 expression in the tumor bulk when compared to the adjacent brain tissue (Fig. 5). The treatment with NE-siRNA CD73R led to a decrease of CD73 expression in glioma-bearing rats when compared to control, confirming the ability of the formulation to delivery CD73 siRNA sequences, resulting in CD73 silencing in vivo. Additionally, qualitative analysis indicates a decrease of CD31/PECAM-1-positive cells, which is suggestive of decreased angiogenesis in NE-siRNA CD73R-treated animals when compared to control (Fig. 5). In accordance with to CD73 silencing, treatment with NE-siRNA CD73R in glioma-bearing rats resulted in dramatic ~96% (0.22 ± 0.04 μM) and ~85% (0.07 ± 0.08 μM) decreases of ADO and INO in CSF, respectively, when compared to control (ADO 4.87 ± 3.73 μM; INO 0.51 ± 0.48 μM; Fig. 6). This data show that CD73 downregulation and nasal administration using nanostructured delivery systems may be an interesting approach for glioblastoma treatment.

NE-siRNA CD73R Treatment Does Not Induce Toxicity in Glioblastoma-Bearing Rats

The safety of the treatment with NE-siRNA CD73R in glioma-implanted rats was determined. As shown in Fig. 7 (panel a), the treatment with NE-siRNA scramble or NE-siRNA CD73 did not decrease the percentage of weight gain

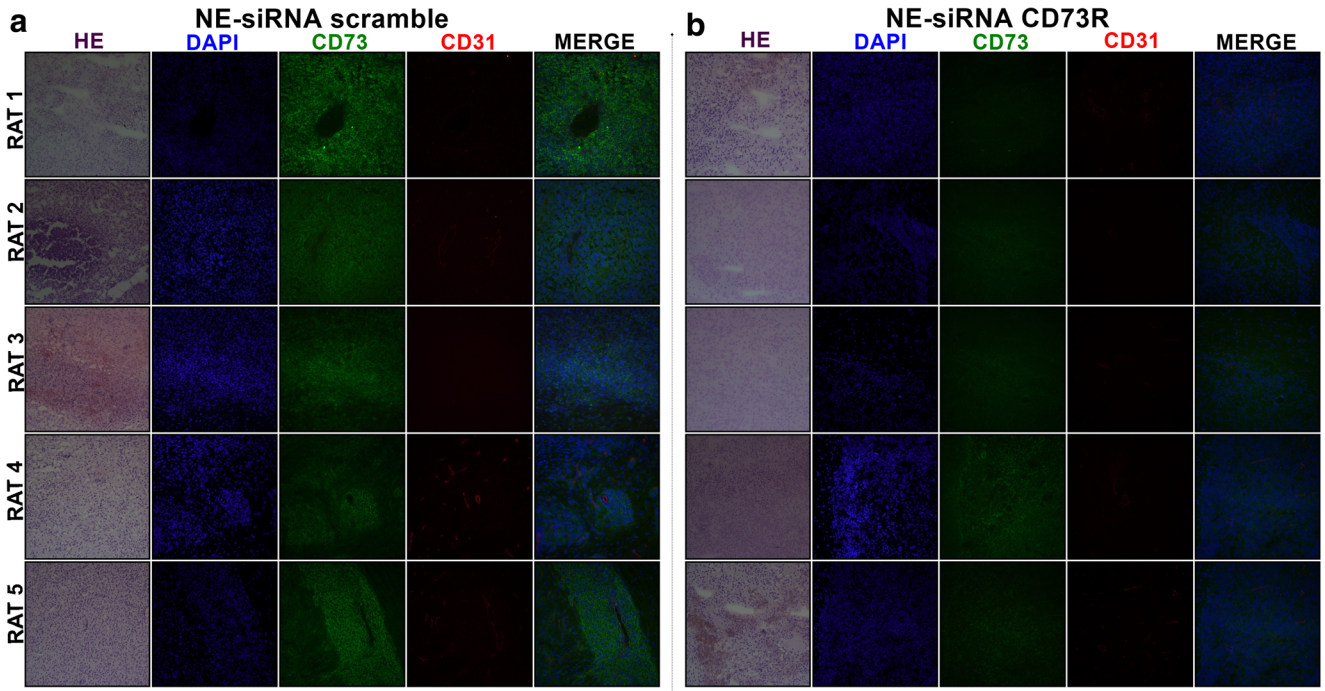

Fig. 5 NE-siRNA CD73R treatment promotes in vivo CD73 knockdown in a preclinical model of glioblastoma. Following 5 days of glioma implant, rats were treated with NE-siRNA complexes (10 μg/kg, 12/12 h). Following 15 days of treatment, IHC analysis of implanted gliomas from NE-siRNA scramble or NE-siRNA CD73R treated groups was performed. The fluorescent staining is as indicated: blue (nucleus-DAPI); green (CD73); red (CD31/PECAM-1). Glioma-bearing rats treated with NE-siRNA CD73R exhibited a decreased number of CD73 and CD31/PECAM-1 immunopositive cells when compared to NE-siRNA scramble treated rats. Images were taken at 200× magnification ($N = 5$ animals). NE-siRNA scramble = nanoemulsion complexed with GFP siRNA; NE-siRNA CD73R = nanoemulsion complexed with rat CD73 siRNA

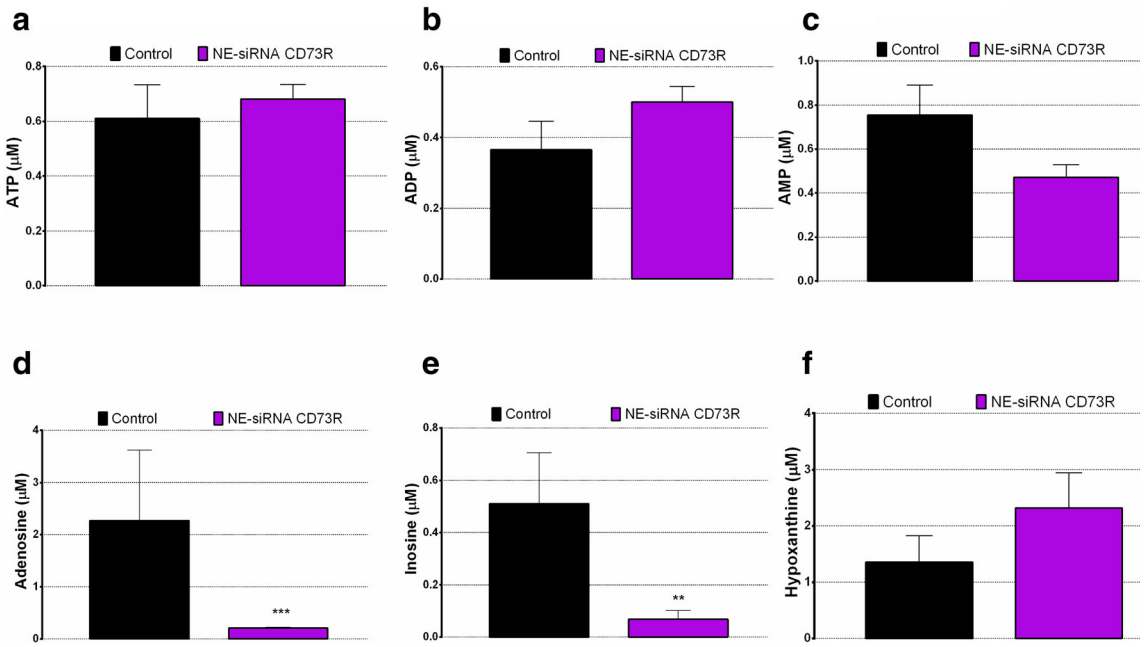

Fig. 6 NE-siRNA CD73R treatment decreases ADO and INO levels in the liquor of glioma-bearing rats. Following 5 days of glioma implant, rats were treated with NE-siRNA complexes (10 μg/kg, 12/12 h) for further 15 days. The liquor was collected and the nucleotide/nucleoside levels were determined by HPLC, as indicated. Values represent the mean ± SD of at least eight animals per group. Data were analyzed by Test T. ***, **significantly different from control (NE-siRNA scramble group) ($p < 0.001$ and < 0.001, respectively). NE-siRNA scramble = nanoemulsion complexed with GFP siRNA; NE-siRNA CD73R = nanoemulsion complexed with rat CD73 siRNA

of the animals. In addition, no alterations were observed in either hematological parameters (Table 3), serum ALT/AST activities, creatinine, urea levels (Table 4), or histology of the lung after 15 days of treatment (Fig. 7b), indicating that the treatment did not affect liver, kidney, and bone marrow functions. Finally, rat behavior evaluation using the open field apparatus indicated that NE-siRNA CD73R-treated animals exhibited an increase of 20% of crossings when compared to control, which may indicate a greater capacity of locomotion, suggesting a better prognosis and a re-establishment of brain functions of these animals (Fig. 7c). The treatment with cationic NE did not alter other behavior parameters related to anxiety (Fig. 7d–f), neither were alterations on social behavior or convulsions on animals observed (data not shown). In addition, the treatment did not cause alterations in the olfactory capacity of the animals, suggesting no damage to the nasal mucosa (Fig. 7g–h). Taken together, these results suggest that the treatment was safe and well tolerated by the animals.

Discussion

Here we show a novel approach for glioblastoma treatment using CD73 as molecular target and the nanotechnology as a strategy for siRNA delivery to CNS. First, a cationic lipid-based NE aiming nasal delivery of CD73 siRNA was developed and exhibited physicochemical characteristics expected for this kind of formulation. We found out that NE-siRNA CD73 complexes were uptaken by glioma cells, which was followed by a parallel decrease of AMPase activity and cell viability, indicating in vitro CD73 knockdown and the potential of CD73 for control glioblastoma progression. Further experiments showed that NE-siRNA complexes achieved CNS after a single administration via nasal route and, notably, the treatment of glioma-bearing rats with NE-siRNA CD73 decreased tumor volume by 60%. The anti-glioma effect of treatment could be related to in vivo CD73 silencing, as both decreased CD73 expression and reduced ADO levels, the product of its enzymatic activity, were identified. Finally, the developed formulation did not induce in vitro and in vivo toxicity, indicating safety of administration.

Glioblastoma remains a challenge for oncology, and effective therapies are not available for patients [1]. In this regard, previous studies from our group showed that CD73 downregulation using specific CD73 siRNA sequences impaired in vitro and in vivo glioma growth, and increased cancer cell sensitivity to chemotherapy. These effects were partially associated with decreased ADO bioavailability and consequent blockage of A1 receptor-mediated effects [10]. Therefore, our data indicate the important role of CD73 in glioblastoma progression and chemoresistance, pointing to this protein as a target for treatment. Next, aiming to develop a strategy for glioblastoma therapy based on CD73 silencing, cationic NE was developed as an approach to perform siRNA CD73 delivery via nasal route. Indeed, drug delivery to CNS is challenging, and the nasal route provides a non-invasive method

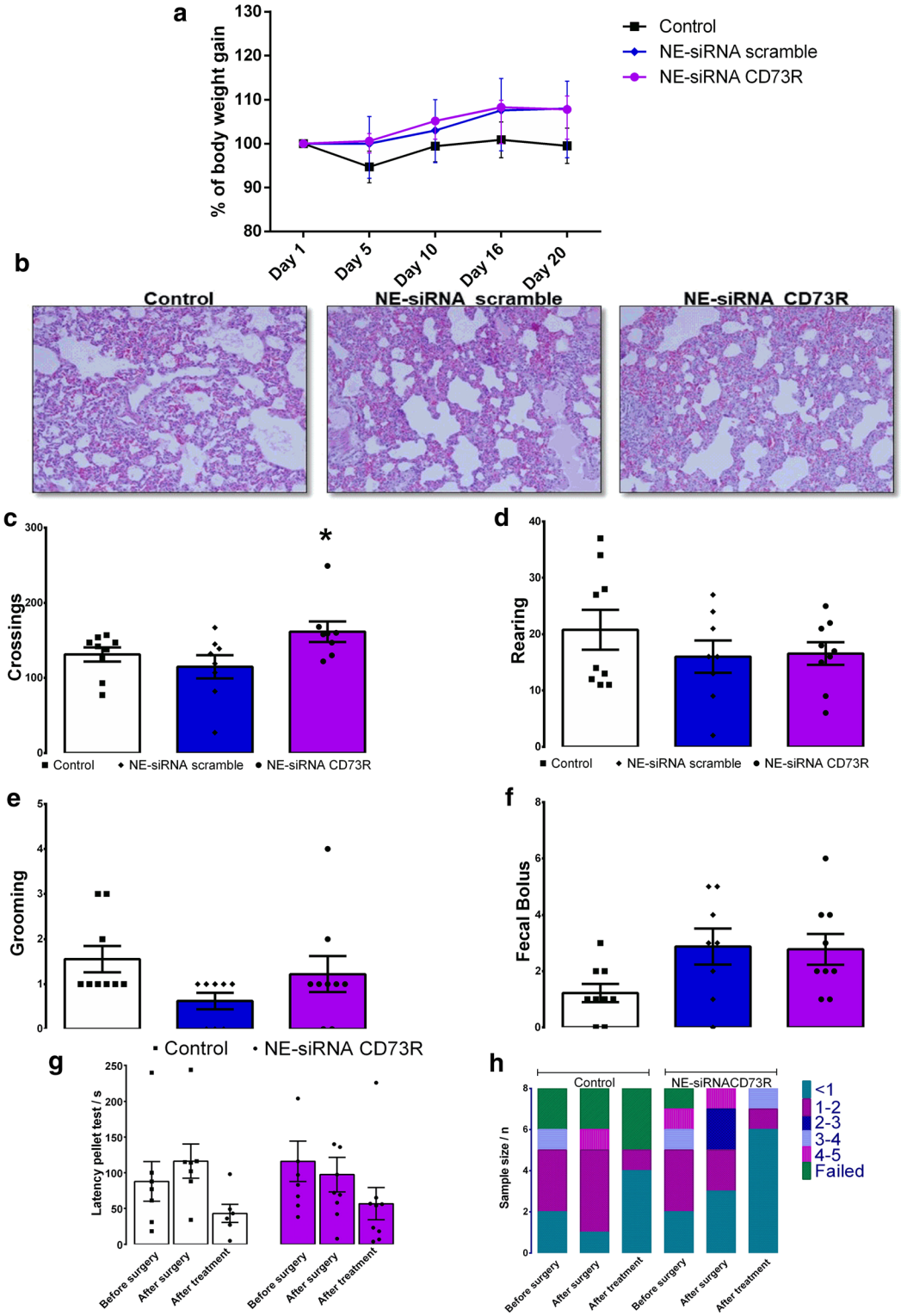

Fig. 7 NE-siRNA CD73R treatment does not induce toxicity in glioma-bearing rats. Following 5 days of glioma implant, rats were treated with either PBS (Control), NE-siRNA scramble, or NE-siRNA CD73R (10 μg/kg, 12/12 h) for further 15 days. **a** Body weight measurements were performed at indicated days in all groups using an analytical balance. **b** Representative HE staining of lung tissue from treated animals demonstrating the absence of histopathological alterations. **c–f** Analysis of behavioral parameters of glioma-implanted rats using Open Field apparatus. Control (white bar), NE-siRNA scramble (blue bar) and NE-siRNA CD73R (purple bar) and **g–h** analysis of olfactory capacity of animals (scale in minutes). Values represent the mean ± SD of at least eight animals per group. Data were analyzed by ANOVA followed by post-hoc comparisons (Tukey's test). *Significantly different from controls (PBS and NE-siRNA scramble group) ($p < 0.05$). NE-siRNA scramble = nanoemulsion complexed with GFP siRNA; NE-siRNA CD73R = nanoemulsion complexed with rat CD73 siRNA

Table 3 Hematological parameters in control or NE-siRNA complexes treated glioma-bearing rats

Control	WBC $10^2/mm^2$	RBC $10^3/mm^2$	HGB g/dl	HCT %	PLT $10^2/mm^2$	PCT %	MCV fm^2	MCH pg	MCHC g/dl	RDW %	MPV fm^2	PDW %	% Lim	% Mon	%Gran	# Lym $10^2/mm^2$	#Mon $10^2/mm^2$	#Gran $10^2/mm^2$
RAT 1	3.3	8.92	16.3	48.6	765	0.48	54	18.2	33.5	14	6.4	10.9	87	8	5	2.8	0.2	0.3
RAT 2	8.2	8.61	16.2	47.7	780	0.51	55	18.8	34	13.9	6.5	10.8	89.3	7.1	3.6	7.3	0.5	0.4
RAT 3	8	9.25	15.7	46.6	722	0.53	50	17	33.8	14.8	7.4	5.1	88.9	7.1	4	7	0.5	0.5
RAT 4	6.3	9.28	16.9	49.4	770	0.50	53	18.3	34.3	13.9	6.6	10.8	90.1	6.6	3.3	5.7	0.4	0.2
RAT 5	4.1	8.54	15.1	44.5	674	0.42	52	17.7	34	14.6	6.3	9.2	89.1	6.6	4.3	3.6	0.2	0.3
Mean	5.9	8.92	16.04	47.3	742	0.49	52	18	33.92	14.2	6.6	9.3	88.8	7.0	4.04	5.28	0.36	0.34
SD	2.2	0.34	0.67	1.90	44	0.04	1.92	0.68	0.29	0.42	0.43	2.48	1.14	0.57	0.65	2.01	0.15	0.11
NE-siRNA CD73R	WBC $10^2/mm^2$	RBC $10^2/mm^2$	HGB g/dl	HCT %	PLT $10^2/mm^2$	PCT %	MCV fm^2	MCH pg	MCHC g/dl	RDW %	MPV fm^2	PDW %	% Lim	% Mon	%Gran	# Lym $10^2/mm^2$	#Mon $10^2/mm^2$	#Gran $10^2/mm^2$
RAT 1	5	8.17	15.2	44.2	837	0.52	54	18.6	34.4	14	6.2	10.7	84	9.7	6.3	4.2	0.4	0.4
RAT 2	7.3	9.39	17.1	50.8	739	0.48	54	18.2	33.6	13.9	6.5	10	79.6	10.4	10	5.8	0.7	0.8
RAT 3	5.6	9.05	16.3	47.5	737	0.47	52	18	34.4	13.9	6.5	11.1	82.2	10.6	7.2	4.6	0.5	0.5
RAT 4	6.1	9.28	16.5	48.7	556	0.44	52	17.8	33.9	14.8	8.1	5.1	84.5	8.2	7.3	5.1	0.4	0.6
RAT 5	5.9	8.87	16.2	47.5	771	0.49	53	18.2	34.1	13.9	6.4	10.6	81.9	10.2	7.8	4.8	0.5	0.5
Mean	5.9	8.95	16.2	47.7	728	0.48	53	18.1	34.08	14.1	6.74	9.5	82.44	9.82	7.72	4.9	0.5	0.56
SD	0.97	0.55	0.79	2.76	117.19	0.03	1.15	0.34	0.39	0.43	0.86	2.78	2.21	1.08	1.59	0.68	0.14	0.17

Glioma-bearing rats were treated via nasal route with NE- siRNA scramble (control of silencing) or NE-siRNA CD73R (10 μg/kg; 12/12 h; 15 days). Hematological parameters were determined in blood collected with anticoagulant using hemocell counter. The values represent the mean ± SD. Data are analyzed by ANOVA followed by post-hoc comparisons (Tukey's test)

NE-siRNA scramble: nanoemulsion complexed to GFP siRNA; NE-siRNA CD73R: nanoemulsion complexed to rat CD73 siRNA

Table 4 Tissue damage serum markers in control or NE-siRNA complexes treated glioma-bearing rats

	Control	NE-siRNA scramble	NE-siRNA CD73R
AST (U/L)	292.4 ± 29.6	302.7 ± 63.3	298.1 ± 31.5
ALT (U/L)	30.4 ± 10.5	28.6 ± 11.2	37.2 ± 7.1
Urea (mg/dL)	52.0 ± 5.1	58.8 ± 9.6	55.2 ± 8.8
Creatinine (mg/dL)	11.6 ± 2.6	10.8 ± 1.6	12.0 ± 3.9

Glioma-bearing rats were treated via nasal route with PBS (control group); NE- siRNA scramble (control of silencing) or NE-siRNA CD73R (10 μg/kg; 12/12 h; 15 days). Tissue damage markers were determined in blood serum using commercial kits. The values represent the mean ± SD. Data are analyzed by ANOVA followed by post-hoc comparisons (Tukey's test)

NE-siRNA scramble: nanoemulsion complexed to GFP siRNA; NE-siRNA CD73R: nanoemulsion complexed to rat CD73 siRNA; NE-siRNA CD73H: nanoemulsion complexed to human CD73 siRNA

to overcome the BBB with several advantages, including fast onset of action, avoidance of intestinal and hepatic drug metabolism, and easy administration [31, 33, 36]. The use of nanoparticles may improve nose-to-brain drug delivery, since they are able to protect the encapsulated drug from biological and/or chemical degradation, and from extracellular transport [30, 32, 40]. The main physicochemical characteristics of NE and complexes obtained in this study were in accordance with those reported in previous studies using NE obtained through microfluidization under similar conditions [40, 47]. The correlation between the increase in charge ratio and the efficiency of oligonucleotide complexation corroborates data already reported in the literature [48–51]. In addition, an efficient system for CD73 siRNA in vitro and in vivo delivery was developed. The complexes at + 4/− 1 charge ratio were internalized by glioma cell in culture and efficiently knockdown CD73 expression, as evidenced by the decrease in AMPase activity but no change in CD73 activity was observed in healthy cells, perhaps because AMPase activity is 30-fold lower when compared to glioma cells. Data from the literature showed higher charge ratios may provide higher transfection efficiency results, since the complex mediated efficient interactions between cells and oligonucleotides [37, 47, 50, 51]. This data show similar silencing efficiency to that obtained with the commercial reagent used for transfection [10]. Interestingly, our results also showed that in vivo nasal administration was efficient to permeate the formulation through the olfactory epithelium, resulting in nanocomplexes reaching rat brain and blood serum following 6 h of a single administration. Our results corroborate a previous study, which demonstrated that cationic NE increases the bioavailability and the residence time of siRNA sequences in the brain when compared to free siRNA administration. Such characteristics may result from the mucoadhesive potential of NE, and its increased

endocytosis due to the small droplet size, which facilitates the passage through the cell barriers [49].

In addition, we demonstrated that NE-siRNA CD73 treatment via nasal route significantly decreased in vivo glioma growth. The therapeutic regimen of administration of 12/12 h was elected based on biodistribution analysis and aimed a sustained and constant CD73 silencing, since studies have shown that keeping CD73 expression and extracellular ADO availability consistently low are important factors for increasing a therapeutic effect [52]. In accordance with in vivo CD73 knockdown, the expression of this protein in the tumor bulk, as well as of ADO levels in CSF, were decreased by NE-siRNA CD73 treatment. We speculate that the decrease of CD73 expression could impair glioblastoma growth mainly by impacting events of cell migration/adhesion/proliferation and by reversing an immunosuppressive microenvironment. Indeed, reports have demonstrated that CD73 is important for tumor migration and invasion, by modulating expression of MMP-2 and vimentin [10] and that ADO, the product of AMPase activity of CD73, is an outstanding immunomodulator, promoting both cancer cell proliferation/survival and immunosuppression, by suppressing cytokine production and proliferation of both CD8+ and CD4+ T cells and regulating myeloid-derived suppressor cells (MDSCs) and macrophages such as M2 [12, 17]. Finally, the treatment was safe and the animals did not exhibit alterations in either biochemical, hematological, histological, or behavior parameters.

In conclusion, we have developed a cationic lipid-based nanoemulsion as a siRNA delivery system for downregulating the *CD73* gene, which has implications in glioblastoma progression. The set of results further suggests a novel pathway that can be exploited for the treatment of brain tumors, which is suitable for in vivo administration, allowing repeated applications and sustained silencing of a target protein, thus resulting in superior therapeutic outcome. Further investigations associating this strategy with the standard therapy for glioma are necessary to improve the therapeutic potential of NE associated with CD73 siRNA.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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