CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRALOVÉ

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

Gene silencing of ICAM-1 by lysine modified oligonucleotides

(Thesis)

in cooperation with

UNIVERSITY OF VIENNA

DEPARTMENT OF MEDICINAL CHEMISTRY

Supervisors:

Prof. Dr. Christian R. Noe

Mag. Martina Stessl

Dr. Johannes Winkler

Doc. PharmDr. František Štaud Ph.D.

Hradec Králové 2009   Mgr. Aneta Kocourková

I here declare that this thesis is an original author's copy, worked out by myself. All literature and other sources used in this thesis are quoted and listed in the literature section.

Mgr. Aneta Kocourková

1.   ABBREVIATIONS

- Ab   antibody
- AC   acrylamide
- AD   Alzheimer's disease
- ALS   amyotrophic lateral sclerosis
- AMD   age-related macular degeneration
- AP   activator protein
- APP   amyloid precursor protein
- ASC   antistratum corneum
- ASO   antisense oligonucleotides
- BACE1   β-secretase
- BBB   blood-brain barrier
- BMEC   brain microvascular endothelial cells
- BSA   bovine serum albumine
- CAM   cellular adhesion molecule
- CDAI   Crohn's disease activity index
- CHAPS   3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
- CNS   central nervous system
- CP   choroid plexus
- CPG   Controlled Pore Glass
- CSF   cerebrospinal fluid
- CVO   circumventricular organ
- DNA   deoxyribonucleotide acid
- DTT   dithiothreitol
- EAE   encephalomyelitis
- ECV   endothelial cell line
- EDTA   ethylene diamine tetraacetic acid
- EMCV-IRE5   encephalomyocarditis virus-internal ribosome entry site
- FANA   fluoro arabinonucleic acid
- GLUT-1   glucose transporter type 1
- HCV   hepatitis C viral
- HIV   human immunodeficiency virus
- HPV   human papillomavirus
- HRP   horseradish peroxidase
- HUVEC   human umbilical vein endothelial cells
- ICAM   intercellular adhesion molecule
- IFN   interferon
- Ig   immunoglobulin
- IL   interleukin
- IMDM   Iscove's Modified Dulbecco's Media
- IRAK   protein kinase
- LIPO   lipofectamine
- LNA   locked nucleic acid
- M   matrix
- miRNA   microRNA

- MS   multiple sclerosis
- NA   neuraminidase
- NBS   newborn calf serum
- NF-κB   nuclear factor-kappa B
- NP   nucleocapsid protein
- NS   non-structural protein
- ODN   oligodeoxynucleotide
- OM   optimem
- PA   a component of the RNA transcriptase
- PAGE   polyacrylamide gel electrophoresis
- PB1   viral polymerase B1
- PB2   viral polymerase B2
- PBS   phosphate-buffered saline
- PECAM   platelet-endothelial cell adhesion molecule
- PIC   protease inhibitor cocktail
- PIP3   phosphatidylinositol 3,4,5-trisphosphate
- PNA   peptid nucleic acid
- PO   phosphodiester
- PS   phosphorothioate
- RISC   RNA induced silencing complex
- RNA   ribonucleotide acid
- RNase H   ribonuclease H
- RNAi   RNA interference
- RNAse H   ribonuclease H
- RSV   respiratory syncytial virus
- shRNA   short hairpin RNA
- SDS   sodium dodecyle sulphate
- siRNA   short interfering RNA
- TBE   tris borate EDTA
- TEAA   triethylammonium acetate
- TETD   tetraethylthiuramidsulphide
- TNF   tumor necrosis factor
- Tris   trishydroxymethylaminomethane
- VCAM   vascular cell adhesion molecule
- VEGF   vascular endothelial growth factor
- VEGFR   VEGF receptor
- 1U*   one 2'-O-lysyl-aminohexyl uridine modification
- 2U*   two 2'-O-lysyl-aminohexyl uridine modifications
- 3U*   three 2'-O-lysyl-aminohexyl uridine modifications
- 2'MOE   2'-O-methoxyethyl

2. INTRODUCTION

2.1. Blood-brain barrier (BBB)

The BBB controls the passage of molecules from the blood into the central nervous system (CNS). It is a physical barrier between local blood vessels and CNS, protecting the brain from many toxins and chemicals having entered the peripheral blood system. Its function is the maintenance of the homeostatic environment of the brain. The BBB also shows clinical importance as it is disrupted in many inflammatory neurological disorders in which an excess of pro-inflammatory cytokine and/or reactive oxygen species are implicated (1).

2.1.1. Anatomy

Three main barrier layers [see Figure 1] at the interface between blood and tissue protect the CNS: the endothelium of brain capillaries, the choroid plexus (CP) epithelium and the arachnoid epithelium (2).
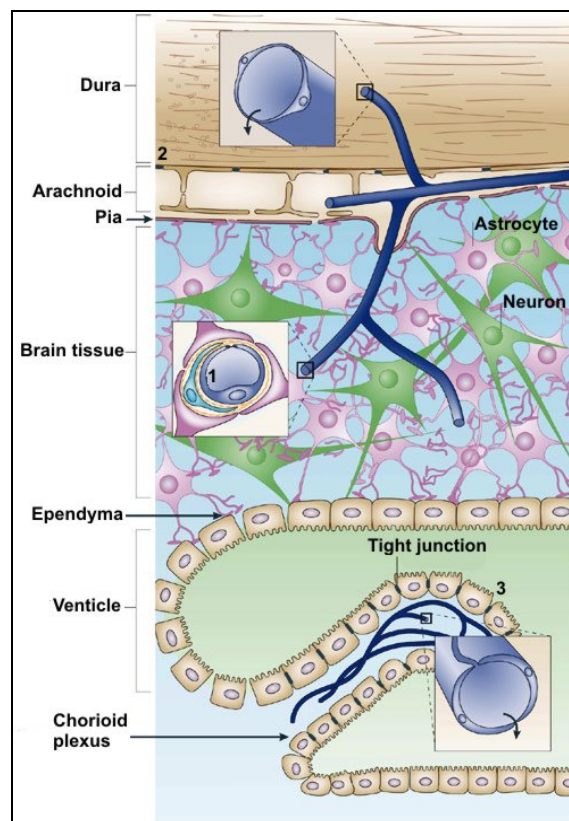
[Figure 1] Scheme of BBB the brain endothelium [1], the arachnoid epithelium [2], the choroid plexus epithelium [3]. Adapted from Segal MB and Zlokovic (3).

The brain endothelium forms the blood-brain barrier. The arachnoid epithelium contains meninges pia and dura. The choroid plexus secrets the cerebrospinal fluid (CSF), which flows through the ventricles, and out to the subarachnoid space, before draining via several routes back into veins. The ependyma lining the ventricles, and the pia at the brain surface facing the subarachnoid CSF, are leaky over most of their surface. In circumventricular organs (CVOs), containing neurons specialized for neurosecretion and/or chemosensitivity, the endothelium is leaky. This allows tissue-blood exchange, but as these sites are separated from the rest of the brain by an external glial barrier, and from CSF by a barrier at the ependyma, CVOs do not form a leak across the BBB (3).

It is clear that neural barrier layers can be composed of a variety of cell types [see Figure 2], both endothelial and epithelial. At brain capillaries (the largest surface area blood–CNS interface) numbers of cell types are associated with the endothelial layer. Pericytes are enclosed within the endothelial basal lamina and form the closest associations with endothelium. The end-feet of astrocytes are opposite to the outer surface of the basal lamina. They cover fine processes and lamellae punctuated by gaps. The perivascular space consists of microglia, neuronal terminals, and (in arterioles) smooth muscle cells. In larger vessels, cells of the meninges form a perivascular cuff or sheath sticking out beyond the brain surface. Obviously interactions between many of these cell types can contribute to the properties of the BBB (2).
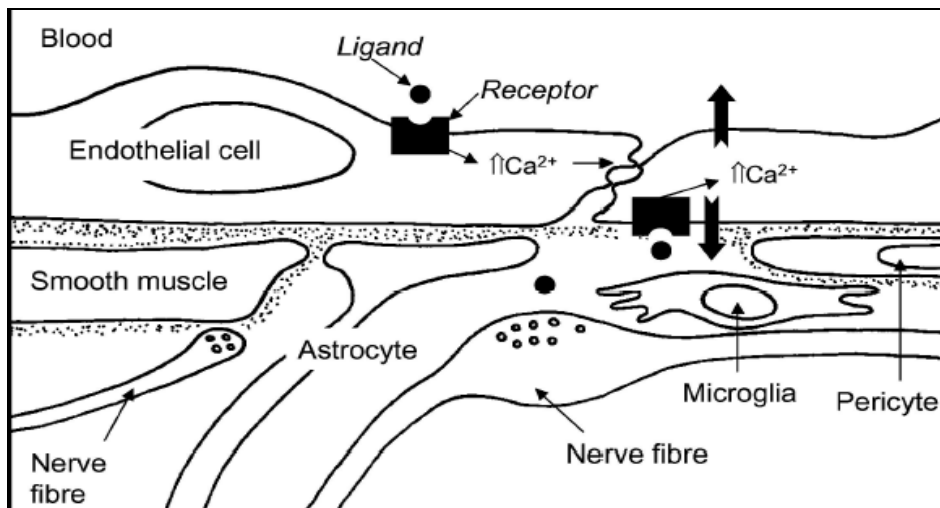
[Figure 2] Cell types forming the blood-brain barier. Adapted from Abbott NJ (2).

2.1.2. Physiology

In the body, walls of capillaries are made up of endothelial cells separated by small gaps. These gaps allow soluble chemicals within tissues to pass into the blood stream, where they can be carried throughout the body, and subsequently pass from the blood into different tissues.

In the brain, these endothelial cells are packed together more tightly, due to the existence of zonulae occludentes (tight junctions) between them, blocking the passage of most molecules from blood to the brain. The blood-brain barrier blocks all molecules except those that cross cell membranes by means of lipid solubility (such as oxygen, carbon dioxide, ethanol, and steroid hormones) and those that are allowed to enter by specific transport systems (such as sugars and some amino acids). Substances with a molecular weight higher than 500Da generally cannot cross the BBB, while smaller molecules often can (4).

2.1.3. Modulation of the blood-brain barrier

Cells of the blood-brain barrier influence processes at the brain endothelium by *induction* (long-term, via regulation of gene transcription) and *modulation* (short-term, generally by protein modification). The brain endothelium has been shown to be modulated by agents via receptor-mediated processes. These agents increase permeability by opening the tight junctional (paracellular) pathway [see Table 1]. Many of the active agents are inflammatory mediators, leading to the hypothesis that BBB modulation plays an important role in CNS inflammation. There is good evidence that, when inflammation is a part of a controlled process it can be beneficial e.g. speeding clearance of infection and promoting recovery (5).

Circulating cytokines produced in response to peripheral damage or infection do not generally cause barrier opening, unless accompanied by CNS pathology. The mature brain endothelium may appear to be designed to limit the inflammatory response. The reason may be to reduce entry of potentially damaging leucocytes (including monocytes/macrophages) capable of disrupting established neural networks. Nevertheless, transient increase in BBB permeability has been demonstrated following application of a number of chemical agents (2).

Humoral agents reported to increase BBB permeability
Bradykinin, serotonin (5HT), histamine, thrombin
Purine and pyrimidine nucleotides: ATP*, UTP, ADP, AMP
Endothelin-1 (ET-1)*
Substance P
Glutamate*, Quinolenic acid
Platelet activating factor (PAF)
Arachidonic acid, prostaglandins, leukotrienes
Cytokines: Interleukins IL-1$\acute{a}$, IL-1$\hat{a}$,
IL-2, IL-6*; tumour necrosis factor-$\acute{a}$. (TNF$\acute{a}$)
Macrophage inflammatory proteins: MIP-1, MIP-2*
Complement-derived polypeptide C3a-desArg
Nitric oxide*, free radicals

Note: Those asterisked have been shown to be released by astrocytic glia.

[Table 1] BBB permeability increased by humoral agents. Adapted from Abbott NJ (1).

Short-term barrier opening might bring certain benefits, including influx of plasma components such as antibodies and growth factors to combat infections and facilitate repair. Interestingly, a small percentage of brain vessels even in normal brain (2–5%) may show signs of tight junction opening. This leads to the possibility that in normal physiology, the barrier may be opening transiently and in a punctuate fashion; in such a way that overall brain homeostasis is not significantly compromised. That this may be a normal process adds support to the use of technologies for opening BBB for drug delivery, using either osmotic opening or pharmacological manipulation, since they may be seen as commandeering an endogenous mechanism (2).

2.1.4. Diseases affecting the blood-brain barrier

The selective permeability of blood-brain barrier represents protection of the brain from common infections, thus infectious diseases of the brain are normally very rare. However, as antibodies are too large to cross the blood-brain barrier, infections of the brain, when they do occur, can be very serious and difficult to treat.

Meningitis is an inflammation of the membranes which surrounds the brain and spinal cord. These membranes are also known as meninges. Meningitis is most commonly caused by

infections with various pathogens. When the meninges are inflamed, the blood-brain barrier may be disrupted, leading to an increase of penetration of various substances (including antibiotics) into the brain. As therapeutic agents third or fourth generation cephalosporins are preferred (6).

Multiple sclerosis (MS) is considered to be an auto-immune disorder in which the immune system attacks the myelin protecting the nerves in the central nervous system. Normally, a person's nervous system would be inaccessible for the white blood cells due to the blood-brain barrier. However, it has been shown using Magnetic Resonance Imaging that, when a person is undergoing an MS "attack," the blood-brain barrier has broken down in a section of his/her brain or spinal cord, allowing white blood cells (T lymphocytes) to enter and destroy the myelin. It has been suggested that, rather than being a disease of the immune system, MS is a disease of the blood-brain barrier. There is no known definitive cure for multiple sclerosis. However, several types of therapy have proven to be helpful. Different therapies are used for patients experiencing acute attacks, for patients who have the relapsing-remitting subtype, for patients who have the progressive subtypes, for patients without a diagnosis of MS who have a demyelinating event, and for managing the various consequences of MS attacks. Treatment is aimed at returning function after an attack, preventing new attacks and preventing disability. Currently only relapsing-remitting multiple sclerosis has Food and Drug Administration approved treatments. These are interferons: Interferon beta-1a (Avonex® and Rebif®) or beta-1b (Betaseron® [in Europe and Japan Betaferon®]). Interferons are medications derived from human cytokines which help regulate the immune system. Glatiramer acetate (Copaxone®, a mixture of polypeptides) may protect important myelin proteins by substituting itself as the target of immune system attack. Mitoxantrone is effective but is limited by cardiac toxicity (7).

Late (second)-stage neurological trypanosomiasis (sleeping sickness) is a condition, in which trypanosoma protozoa have crossed the blood-brain barrier. The organo-arsenical melarsoprol (Arsobal®) was developed in the 1940s, and is effective for patients with second stage sleeping sickness, no matter which parasite is the cause. However, 3 - 10% of those injected have reactive encephalopathy (convulsions, progressive coma, or psychotic reactions), and 10 - 70% die; it can cause brain damage in those that survive the encephalopathy. However, due to its effectiveness, melarsoprol is still used today.

Eflornithine (difluoromethylornithine) is the only currently available medicine which can treat the second neurological phase of *T.b. gambiense* (8).

Progressive multifocal leukoencephalopathy is a rare disease involving plaques of demyelination in the central nervous system caused by the polyomavirus JCV (papovavirus). Oligodendrocytes from these lesions, which are responsible for the synthesis and maintenance of the myelin sheath around neurons, are productively infected by JCV. It affects immune-compromised patients and is usually seen on patients suffering from AIDS and/or elderly patients. In some cases, the disease slows or stops if the patient's immune system improves; some patients have been able to survive for several years, with the advent of highly active antiviral therapy like cidofovir, interleukin-2 or cytarabine (9).

De Vivo disease (also known as GLUT1 deficiency syndrome) is a rare illness caused by inadequate transport of glucose across the barrier, resulting in mental retardation and other neurological problems. Genetic defects in glucose transporter type 1 (GLUT1) appears to be the main cause of De Vivo disease (10, 11).

Alzheimer's disease (AD) is a neurodegenerative disorder caused by a breakdown in the BBB allowing to plasma with amyloid-β to enter the brain. Amyloid- β accumulates to the surface of astrocytes. Eventually the astrocyte is disintegrated, overwhelmed, leaving behind the plaque. AD is the most common form of dementia including memory impairment, decline in mental functions and personality changes. There exists no cure for AD or to slow its progression, but there is available medicine that may help improve mental function of people with AD. Cholinesterase inhibitors (donezepil, rivastignine and galantamine) increase the level of acetylcholine, which improve cognitive symptoms. Neuroleptics, antidepressants, anxiolytics and sleeping medications are intended for behavior and sleeping problems of the patient (12, 13).

2.2. Adhesion molecules (CAMs)

Cellular adhesion molecules were initially defined as cell surface structures mediating cell interactions. These molecules play an essential role in inflammation and immunosurveillance

processes. CAMs involved in immune responses have been classified into three families according to their structure: selectins, immunoglobulin (Ig) superfamily and integrins (1).

2.2.1. Selectins

Selectins are a group of type I transmembrane glycoproteins with a unique and characteristic extracellular region composed of an amino-terminal lectin domain that binds to a carbohydrate ligand, an epidermal growth factor like domain, and short consensus repeat units that are identical to domains found in complement binding proteins. Selectins (E-selectin, P-selectin, L-selectin) mediate rolling of leukocytes on the endothelium of postcapillary venules. E-selectin is synthesized after stimulation by cytokines and expressed on the endothelial cell membrane after several hours. P-selectin is presented on granule membranes in endothelial cells and platelets. Therefore, it can be expressed on the outer cell membrane immediately after cell activation by stimulants such as thrombin or histamine. The target cells of both E- and P-selectin are neutrophils and monocytes. L-selectin is presented on lymphocytes, neutrophils, and monocytes. After cellular activation, it is shed from the cell membrane by proteolytic cleavage (14).

2.2.2. Integrins

After rolling of the leukocyte on the endothelial surface has arrested its flow, leukocyte integrins are activated by chemokines, chemoattractants, and cytokines. Integrins are transmembrane cell surface proteins. The CD18 or β2 integrins are restricted to leukocytes and bind to their counter receptors of the immunoglobulin gene superfamily. They share a common β chain and 3 distinct α chains (CD11a, CD11b, or CD11c). Their surface expression is increased by agonists such as TNF-α (tumor necrosis factor-alpha) and after adhesion to E-selectin. Leukocyte integrins are involved in the firm adherence of the leukocyte through binding to the endothelial Ig gene superfamily molecules. Leukocytes and monocytes also express the integrin α4β1 (very late antigen-4, CD49d), which binds to VCAM-1 (vascular cell adhesion molecule-1) and to ligands from the subendothelial matrix (14).

2.2.3. Immunoglobulin (Ig) superfamily

Firm adhesion of leukocytes to the endothelial cells as well as leukocyte activation is mediated by receptors of the Ig gene superfamily. This family contains 5 molecules that are expressed by endothelial cells: intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2), VCAM-1, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and the mucosal addressin. The CAMs are involved in leukocyte adhesion at relatively low shear forces; they cause a stronger attachment of leukocytes to the endothelium than selectins. ICAM-1 (CD54) is continuously presented in low amounts on the cell membranes of endothelial cells, leukocytes, epithelial cells, and fibroblasts. Its expression is greatly increased by stimulation of cytokines. ICAM-2 (CD102) is a membrane receptor of endothelial cells that does not increase after stimulation, whereas VCAM-1 (CD106) expression on endothelial cells is induced by TNF-α and interleukin-1 (IL-1). PECAM-1 (CD31) plays an important role in the attachment of endothelial cells to each other, in leukocyte adhesion, and particularly in transmigration across the endothelium. Its surface expression on endothelial cells is not increased by cytokines (14).

2.3. ICAM-1

ICAM-1 is a 95-105-kDa cell surface glycoprotein, which has first been identified by the ability of a monoclonal antibody to block phorbol ester-induced aggregation of a B-cell line. ICAM-1 belongs to the immunoglobulin gene superfamily and contains five immunoglobulin domains [see Figure 3]. A motive within domains 1 and 3 of ICAM-1 is critical for its binding to β2 integrins. Particularly ICAM-1 is described as a ligand for the membrane-bound integrin receptors called leukocyte function associated molecule 1 (CDlla, CDl8), Mac-1 (CD11b/CD18) and CD 43 on leukocytes. ICAM-1 spans to the cell membrane and contains only a short cytoplasmatic tail. ICAM-1 is expressed at low basal level in fibroblasts, leukocytes, keratinocytes, endothelial and epithelial cells but is up-regulated in response to a variety of inflammatory mediators. Expression of ICAM-1 is inducible by a number of cytokines including IL-1, TNF-α and interferon-7 (IFN-7). The broad tissue and cellular distribution of ICAM-1 suggests that it is not only involved in the migration of leukocytes out of the vasculature, but may play a more extensive role in immune responses. Additional roles for ICAM-1 include localisation of leukocytes to the area of inflammation in extravascular

spaces, enhanced recognition of antigen-presenting cells by T-lymphocytes, formation of lymphocyte germinal centers, enhancing natural killer cell response, and differentiation of thymocytes. In addition, ICAM-1 is the receptor for more than 90% of the rhinovirus serotypes (1, 15, 16, 17).
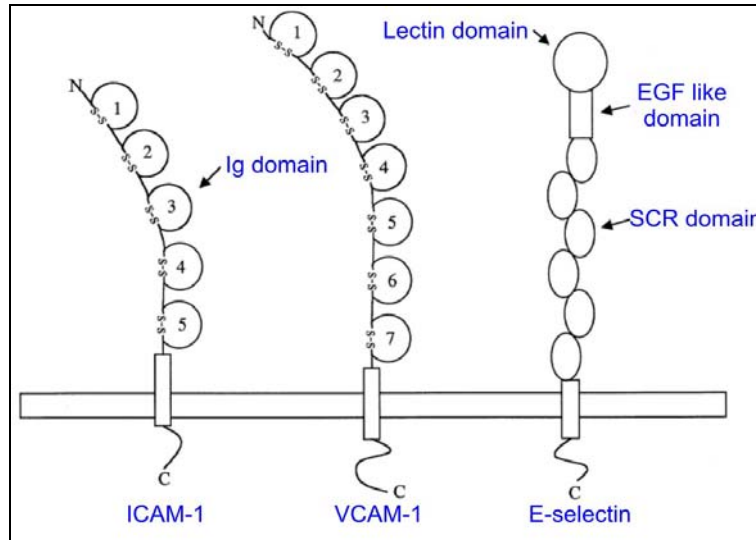
[Figure 3] Structure of ICAM-1 to compare with VCAM-1 and E-selestin. Adapted from Lee SJ and Benveniste EN (18).

The human *ICAM-1* gene has been cloned and its 5'-regulatory region isolated and sequenced. The promoter region of *ICAM-1* gene contains consensus sequences known to bind transcription factors. Among them Sp-1, AP-1 (activator protein 1), AP-2 and NF-κB (nuclear factor-kappa B) responsive elements were identified, as well as retionic acid responsive elements, and the core sequence for binding to the Ets family of transcription factors. The *ICAM-1* gene consists of seven exons separated by six introns (1).

2.3.1. Induction of ICAM-1 gene expression

ICAM-1 is widely distributed in leukocytes, endothelial cells and many other tissues. Many factors are able to up-regulate the expression of this molecule such as proinflammatory cytokines, which strongly increase the expression level of ICAM-1 (1).

Up-regulation of ICAM-1 expression by TNF-α, interleukins and interferons
Up-regulation of ICAM-1 expression with TNF-α is observed in brain microvascular endothelial cells (BMEC). It was shown that specific flanking sequences for the NF-κB

16

response elements are necessary for TNF-α-induced *ICAM-1* gene expression. Recently it was found that the entymeprotein kinase C-zeta can induce TNF-α induced NF-κB binding to the *ICAM-1* gene transcription. The binding of TNF-α to its two receptor results in recruitment of signal transducers activating three different effectors, which subsequently leads to the activation of caspases and of the transcription factors, AP1 and NF-κB. Using anti-TNF monoclonal antibody, ICAM-1 cell surface expression could be slightly reduced in the case of previous TNF stimulation of endothelial cells (1).

Ibuprofen also inhibits pyrogen-dependent adhesion of leukocytes to these endothelial cells. IL-4 had the same effect, reducing IL-1β-induced ICAM-1 up-regulation on the surface, of human umbilical vein endothelial cells (HUVEC) (14).

The *ICAM-1* gene expression is also up-regulated by IL-1 in HUVEC. Recently, using microarray analysis, the induction of ICAM-1 mRNA by IL-8 was demonstrated in HUVEC. IL-1 binds to a type I receptor and a heterodimer is formed by association of IL-1 receptor accessory protein with the ligand receptor complex. Rapidly, IL-1 receptor activates protein kinase (IRAK) that is recruited to the heterodimer and activated. IRAK can interact with TNF-receptor associated factor 6 and results in NF-κB activation and stimulation of gene expression. An antisense human IL-1 receptor associated kinase-2 (IRAK-2) oligonucleotide was shown to inhibit IL-1 stimulated NF-κB activation and ICAM-1 expression in cultured endothelial cells. (11) A lipid soluble vitamin α-tocopherol acts as an antioxidant and also reduces IL-1β-induced up-regulation of ICAM-1 and VCAM-1 on HUVEC indicating that α-tocopherol may work as an anti-inflammatory drug by inhibiting leukocyte-endothelial cell adhesive reactions (19).

IFN-γ is another molecule which was reported to up-regulate ICAM-1 expression in BMEC. Induction of transcription of the *ICAM-1* gene by IFN-γ requires two IFN-gamma activated sites located in the *ICAM-1* promoter (11).

Regulation of ICAM-1 gene expression by lipopolysacharide, phorbol esters and retinoic acid

Further components, which are reported to cause a significant stimulation of ICAM-1 expression are lipopolysaccharide in BMEC, phorbol esters (12-*O*-tetradecanoylphorbol-13-acetate and phorbol myristate acetate) in HUVEC and retionic acid in monocytes cells. These substances were shown to inhibit basal and interferon-γ-induced ICAM-1 expression (1).

Hormonal regulation of ICAM-1 gene expression

Hormones can act directly on the expression of ICAM-1 or modulate the expression up-regulated by cytokines. Insulin-like growth factor-1 activates NF-κB and increases ICAM-1 expression in HUVEC. TNF-α-induced ICAM-1 expression in human brain endothelium is reduced by high doses of methylprednisolone. Glucocorticoid hormones are also anti-inflammatory drugs, which are bale to inhibit the up-regulation of ICAM-1. 17-β-Estradiol also strongly inhibited IL-1β-mediated ICAM-1 induction in HUVEC (1).

ICAM-1 gene expression regulated by cells and viruses

Monocyte adhesion to endothelial cells appears to stimulate their own recruitment via induction of ICAM-1 gene expression. Adherence of monocytes to endothelial cells is known to induce cytokine gene expression: a positive feedback mechanism can be mediated by secretion of IL-1β associated with ICAM-1 and VCAM-1 expressions (20).

2.3.2. ICAM-1 and the blood-brain barrier

The BBB is physiologically defined by low permeability for small hydrophilic molecules and low rates of fluid-phase endocytosis. In addition for the entry of a limited set of nutrients (certain amino acids or glucose) and a restricted number of macromolecules (transferrin, lactoferrin, and insulin) is also allowed. Tight junctions and the transporter P-glycoprotein, which transports small molecules out of the cells, maintain the homeostasis of the brain. If BBB is disrupted, an excess of pro-inflammatory cytokines and/or reactive oxygen species (NO) are induced (1).

Leukocyte traffic into CNS

Leukocyte recruitment to the CNS involves interactions with vascular endothelial cells. It is well known that this occurs within post capillary venules and is dependent on a cascade of precise events involving adhesion molecules. Fast-moving leukocytes in the bloodstream are tethering and rolling [see Figure 4] on the activated endothelium. Selectins induce and support rolling. Chemokines, lipid mediators and other pro-inflammatory molecules presented on the surface of the endothelium activate integrins and allow cells to adhere firmly. Once adherent, leukocytes can migrate across the endothelium and enter the inflamed tissue. ICAM-1 is

involved in the firm adhesion of leukocytes to the endothelium, due to its interaction with the integrin LFA-1 (1).
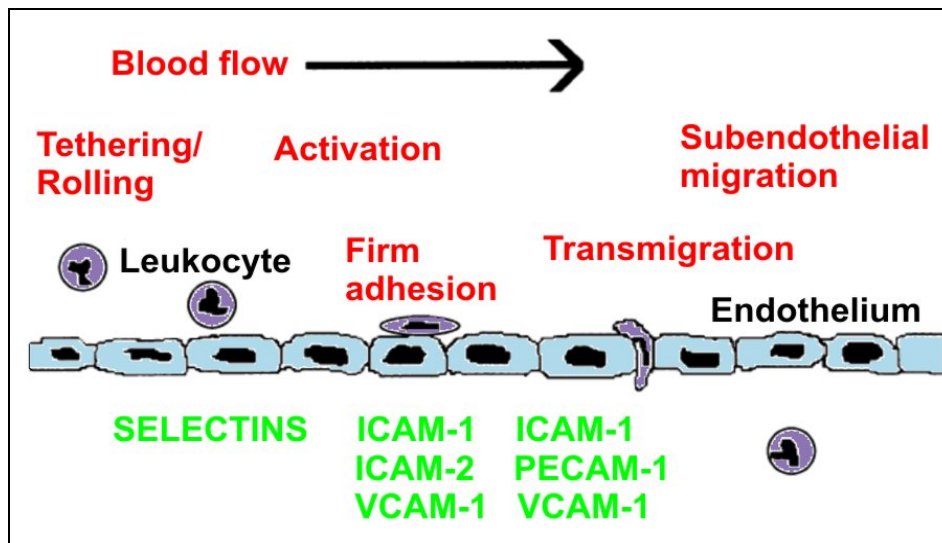
[Figure 4] Adhesion steps of ICAM-1. Adapted from Dietrich JB (1).

In addition, ICAM-1 is essential for transendothelial migration of T cells and is involved in facilitating T lymphocyte migration through the tight junctions of the BBB. Lymphocyte adhesion to the CNS endothelium is low in comparison to non-CNS endothelia, supposable due to a low expression of adhesion molecules or to the high surface charge of the brain endothelium. An early and important event in inflammatory and immune-mediated CNS diseases is the increased lymphocyte traffic across an altered BBB. In healthy tissue, very few leukocytes penetrate the BBB, whereas under pathological CNS conditions, the barrier becomes compromised and an intense infiltration of the CNS by T lymphocytes occurs (21). Up-regulation of ICAM-1 in the vascular endothelium of the CNS is one in many characteristics of various brain inflammations, especially in active experimental autoimmune encephalomyelitis (EAE) lesions (an animal model of multiple sclerosis MS). In EAE and MS the BBB is disrupted resulting in a higher permeability of many substances. Increased expression levels of adhesion molecules as well as interactions are involved in initiation and propagation of autoimmune diseases (1).

Leukocyte mediated breakdown of BBB followed by their recruitment into the CNS is indeed a process characteristic of several CNS disorders, such as MS. MS derived BMEC expressed higher levels of ICAM-1 and especially of LFA-1, a ligand of ICAM-1, in comparison to healthy cells. Thus, the increased expression of ICAM-1 on BMEC correlates with the adhesion and extravasation of leukocytes across the BBB. It is worth noting that most effective therapies against MS interfere with cell adhesion (1). Well-known anti-inflammatory

19

drugs like glucocorticoids inhibit leukocyte migration to sites of human inflammation. It was shown that high-dose of methylprednisolone reduces the cytokine-induced expression of ICAM-1 and VCAM-1 on human BMEC. Methylprednisolone also induces apoptosis in peripheral blood leukocytes. Induction of apoptosis might contribute to the down-regulation of T-cell activity and terminate inflammation in the CNS (22).

ICAM-1 and cerebral malaria

ICAM-1 plays an important role in the pathology of cerebral malaria, for it is thought to be involved in the uptake of Plasmodium falciparum – infected erythrocytes into the brain. Infected red blood cells can bind to the first IgG like a domain of ICAM-1, which functions as a receptor, and the pathogen, can enter the CNS (1).

ICAM-1 and bacterial meningitis

The entry routes of pathogenic microbes into the CNS are diverse and include paracellular and transcellular penetration of cells located in the BBB. Circulating microbial products can also induce a loss of BBB function. Bacteria can enter the cerebrospinal fluid (CSF) and cause meningitis, as it is shown in the case of Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitides. Cell walls of S. pneumoniae, the most common cause of adult meningitis, were found to induce TNF-α, and ICAM-1 expression in BMEC cultures (1).

ICAM-1 and viral infections

Viral infections of the brain are also known to up-regulate cell adhesion of molecules on the brain endothelial cells, astrocytes and microglia. Particularly in the case of human immunodeficiency virus (HIV), leukocyte infiltration is mediated by ICAM-1 and VCAM-1. BBB dysfunction observed in AIDS patients is related to HIV infection of the brain endothelium, which could facilitate virus entry to the CNS, either by infection of the human brain capillary endothelial cells or via entry of HIV-infected leukocytes (23).

Soluble ICAM-1 and MS

Circulating forms of adhesion molecules or soluble receptors may be released from cells as a consequence of activation, and could be used as markers for inflammation in the CNS like in the case of s-ICAM-1. Circulating forms of ICAM-1, ICAM-3, VCAM-1 and L selectin were increased in serum and CSF from most MS patients. These raised levels of circulating ICAM-1 and VCAM-1 are believed to reflect disease exacerbation and BBB disturbance (24).

2.4. Oligonucleotides

2.4.1. History

Nucleic acid compounds, called oligonucleotides, are unmodified or chemically modified single-stranded ribonucleic acid (RNA) or double-stranded deoxyribonucleic acid (DNA) molecules. Commonly they are relatively short (13–25 nucleotides) and represent a unique sequence among targets present in cells. Research in the sequencing of the human genome has led to the use of short fragments as tools to study gene function (25).

The first step towards understanding of genetic information was the discovery of the nucleic acids by F.Miescher in 1869. However, it took long time until biological compounds like the heterocyclic bases adenine, guanine, cytosine and thymine were characterized. At the same time in the twentieth century Ascoli described uracil, which is present instead of thymine in RNA. After the discovery of the sugar components D-ribose and 2-deoxy-D-ribose, classification of RNA and DNA could take place (26).

W. Sutton hypothesized that chromosomes carry the units of inheritance and that their behavior during meiosis is the physical basis of Gregor's Mendel concept of heredity. In 1903 W. Sutton concluded that chromosomes contain units of heredity (now known as genes) and that their behavior during meiosis is random.

Finally in 1953 the key for understanding the mechanism of genetic information transfer was found by J.Watson and F.Crick in their ingenious concept of base pairing. The findings of nucleic acids had impacts on pharmaceutical research. Unwanted processes involving DNA and RNA actions are dominant in cancer and viral diseases. The new field of antimetabolites emerged and resulted in nucleoside drugs still relevant today (aciclovir, zidovudine, didanosine). Antimetabolities, as well as alkylating agents, may be seen as a "first generation" of drugs acting at the genetic level. It is established that apart their function as "fake building blocks" in a nucleic acid chain, their mechanism of action includes the whole enzymatic array controlling nucleic acid synthesis. These drugs are mostly either modifications of nucleobases or nucleosides. Nucleotide drugs, which contain a phosphate moiety attached to the nucleoside, do not appear among the presently used compounds (26).

 In gene therapy genes or specific nucleic acid sequences of genes are the direct target of therapeutic attack. Attention has been paid to somatic gene therapy, in which therapeutically DNA segments are inserted into genomic DNA and are expressed in the human body. They

exert their effects either after transcription at the RNA-level or after the translation at the protein level (26).

Additionally in 1998 Isispharm received acclaim for the first antisense oligonucleotide (ASOs) drug on the market, Vitravene®, for use against cytomegalovirus in AIDS sufferers. Initially the technology looked promising; due to the ability of ASOs to selectively inhibit the expression of its complementary mRNA strand (27) and hence reduce the expression of proteins known to conduce to the disease's pathology. So far many ASOs have failed to deliver the miracle treatment they initially promised. Problems have included poor penetration through membranes, insufficient delivery to the target site and unspecificity regarding the target (28). The search goes on for appropriate modifications to these molecules to improve their clinical effectiveness.

2.4.2. Antisense oligonucleotides (ASO)

Every gene codes different proteins, which have different functions in the body. Naturally many of these proteins can be wrongly synthesized or over expressed. Gene expression [see Figure 5] occurs in human cell nuclei, where gene is transcribed and continues to the cytoplasm, where translation into the corresponding protein takes place. This metabolism is initiated by a transcription. Transcription is the process where proteins recognize specific DNA sequences and locally denature the double stranded DNA. This allows RNA polymerase (pol II) to transcribe the antisense DNA strand into pre-mRNA. The pre-mRNA is stabilized by 'capping' of the 5' end with a methyl-guanosine. Most pre-mRNA species are polyadenylated which stabilizes the pre-mRNA and is important for transportation of the mature mRNA out of the nucleus. The introns are spliced out before leaving the nucleus to produce mature mRNA, which then can undergo translation into the protein on ribosomes (25).
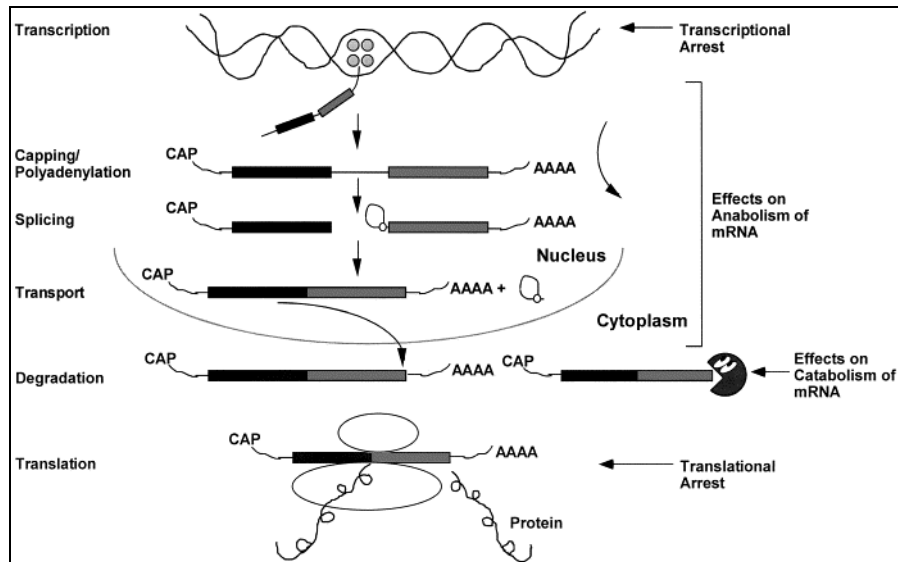
[Figure 5] Gene expression. Adapted from Crooke ST (21).

Double stranded DNA consists of two strands. One of them is named "coding strand" (sense strand). It contains the triplet code for the protein-sequence to be expressed. The other strand is the "complementary strand" (antisense strand), which contains the complementary nucleotides in antiparallel arrangement. It is usually not expressed in the course of a specific gene expression. In a double helix conformation it is not apparent without referring to gene expression, which one is the coding strand. During gene expression, a gene is transcribed from DNA to pre-mRNA, several post-transcriptional modifications turn pre-mRNA into mature mRNA, which is then translated into one or more specific protein splicing variants. In this process the term "sense" connotes the mRNA-sequence of bases that is translated into the protein. The "antisense" sequence contains the complementary bases in an antiparallel configuration. Thus "antisense compounds" can interact with transcribed RNA. "Antisense action" is also used to illustrate the inhibition of mRNA [see Figure 6], mediated by molecules binding to sites formed by tertiary structures of single strand mRNA and interaction of molecules with duplex DNA by sequence specific insertion into the grooves (26).
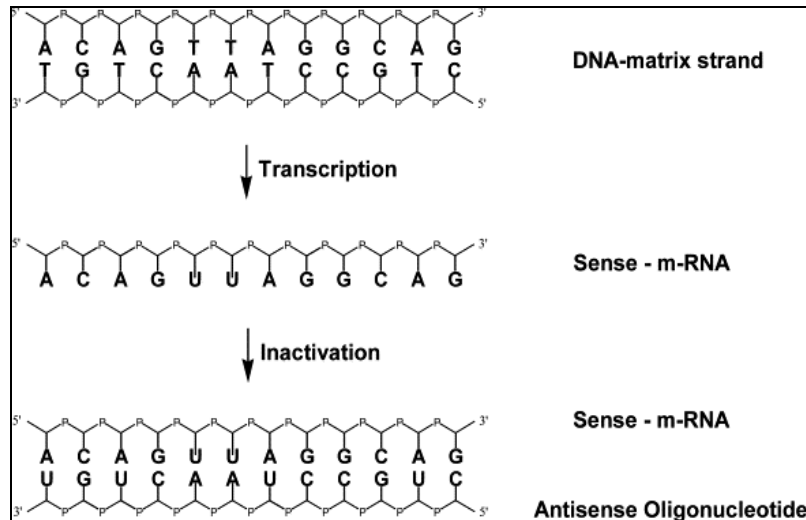
[Figure 6] DNA and RNA regions recognized by an ASO. Adapted from Urban E and Noe CR (29).

The first step to suppress the protein synthesis may be achieved by triple helix formation to blockade the transcription [see Figure 7]. Secondly, ASOs may interfere with processing proteins, which are responsible for the transformation of the primary DNA transcript into the maturated mRNA. Finally, double strand formation between ASOs and the mRNA may disable the transport of mRNA from the nucleus to the cytoplasm (29).
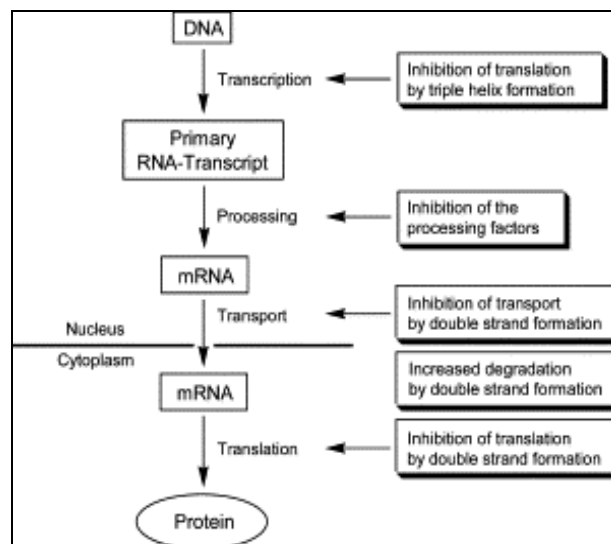
[Figure 7] Inhibition sites of protein synthesis. Adapted from Urban E and Noe CR (29).

2.4.3. Mechanism of action

Binding of oligonucleotides to specific nucleic acid sequences inhibits interaction of the DNA or RNA with proteins or other nucleic acids essential in the intermediary metabolism of the

24

RNA (25). The following are the most investigated mechanisms and those relevant to this project, although others exist.

The most common working mechanism of oligonucleotides is translational arrest, which means binding to the translation initiation codon. The positioning of the initiation codon however, varies considerably. Presently only few studies have shown that oligonucleotides really act by binding to the site for which they were designed (25).

Another mechanism of action includes inhibition of splicing. Oligonucleotides bind with sequences required for splicing, which means excision of introns. Thus oligonucleotides prevent the required cleavage reactions resulting in inhibition of the mature mRNA production (30).

Another mode of action includes disruption of the RNA three-dimensional structure, which provides additional stability for RNA (30).

RNAse H activation

Activation of RNase H is another important characteristic of ASO's, thought to be essential for the efficiency of ASOs. RNase H is an enzyme that hydrolyzes the RNA strand of the RNA-DNA duplex. It has been revealed that ASOs with DNA like properties can activate RNase H (31). However, only little is known about the role of structural features in RNA targets to activate RNase H. Recent studies have shown that a 20mer phosphorothioate complementary to a sequence in the 3'-untranslated region of ICAM-1 RNA inhibits ICAM-1 production in human umbilical vein endothelial cells (32). A 2'-O-methyl analog of this phosphorothioate displays a higher affinity to the RNA but is a less potent inhibitor of ICAM-1. It is therefore more likely that the unmodified phosphorothioate acts by destabilizing the RNA and activating RNase H. Additionally, a 18mer phosphorothioate, complementary to the translation initiation codon of ICAM-1, inhibited production of the protein but caused no RNA-degradation. Therefore two oligonucleotides capable of activating RNase H have different effects depending on the position of the mRNA to which they bind (30).

2.4.4. Factors affecting antisense activity

Current problems with ASOs include lack of cell specific delivery, poor stability against degradation, limited cellular uptake, lack of specificity for the intended target and unknown mechanisms of target deactivation (27). However there is evidence that a reduction or elimination of the charge can enhance cell uptake (33).

Affinity

The two major contributors to binding affinity are hydrogen bonds (via base pairing) and base stacking in the double helix. Thus, affinity increases as the length of the oligonucleotide-receptor complex increases. The theoretical minimum length of an oligonucleotide for selective targeting of a single gene is 12 to 14 nucleotides (25). The real affinity constants are substantially lower than the theoretical ones due to the fact that RNA and oligonucleotides can adopt a variety of secondary and tertiary structures. To avoid aggregation, oligonucleotides containing self complimentary regions or repetitive oligonucleotides should not be used. Thus any modifications made to an oligonucleotide must not significantly interfere with its base pairing ability. This makes the 2'-position and ideal site for modifications such as ligand attachment (34).

Specificity

Specificity goes along with Watson-Crick base pairing. At the genomic level any sequence of 17 residues is expected to occur only once (35). To exploit the theoretical potential for specificity of an oligonucleotide in a therapeutic context, it is necessary to manipulate the length of the oligonucleotide and its concentration at the target.

Critical aspects with regard to administration forms are: sufficient absorption from the site of administration, distribution to tissues, transport into target cells, sufficient residence time and concentration at the site of action to elicit an effective biological response. Throughout this process the oligonucleotide must be resistant to degradation by exo- and endonucleolytic plasma and tissues nucleases. At the intracellular site of action, the oligonucleotide must sequence-specifically bind (hybridize according to Watson-Crick base-pairing rules) to the target RNA that provides inactivation of its function (mode of action). This inactivation may result due to binding to the target sequence or likely by endonucleolytic cleavage of the RNA target strand by RNase H. Another requirement is acceptable toxicity of antisense drug. Toxicity may arise from metabolites generated by nucleolytic degradation, particularly providing modified nucleotides, which may inhibit cellular nucleic acid metabolism (36). Phosphorothioates tend to bind to a wide range of proteins with relatively low affinity but these interactions reduce the therapeutic index less than was expected as the potential toxic effects are 'buffered' (27).

2.4.5. Modifications of ASO's

ASOs are small nucleic acid sequences that are complementary to their target mRNA according to Watson-Crick base pairing. Triple-helix–formation of oligodeoxynucleotides hybridizes to purine-pyrimidine sequences in double-stranded DNA. Formation of stable triple helices can prevent the unwinding of DNA, which is a necessary requirement for the inhibition of mRNA by arresting translation, inhibiting mRNA processing or inducing mRNA degradation via activation of RNase H in the nucleus. The end result of a successful inhibition is the blocked production of the corresponding protein (28, 34). ASO's based on the endogenous phosphodiester backbone of DNA are inclinable to degradation by exo- and endonucleases. Soon after their discovery, modifications [see Figure 8] were composed to the backbone to make them more stable. There have been several generations of ASOs. First generation ASOs (for example the phosphorothioates such as alicaforsen) are based on DNA, whereas second generation molecules have both RNA and DNA characteristics. Second generation ASOs have greater affinity and consequently an enhanced potency over first generation drugs as the target RNA hybridizes more tightly to RNA than DNA (37).
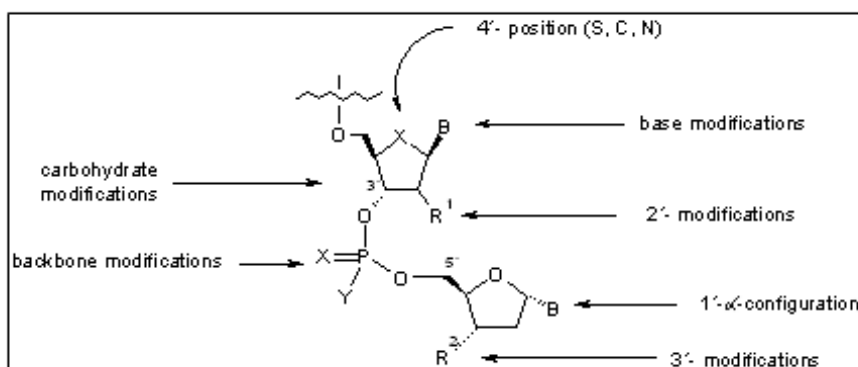
[Figure 8] Chemical modifications of the oligonucleotides.

First generation of ASOs

The most widely characterized phosphorothioates belong to the first generation ASOs; they contain the same structure as DNA except one sulphur atom replaces the non-bridging oxygen in the phosphodiester backbone. The effect of this substitution is that they are with difficulty degraded by endo- and exonucleases. The ability to activate RNAse H made them a promising new class of antisense molecules. They are water soluble, because of their charge (25, 34). A major inconvenience of these ASOs is imperfect sequence specificity and unspecific effects (26).

To this group belongs fomivirsene (Vitravene®), the first approved antisense drug. Other prominent representatives of this group are oblimersen and alicaforsen. Oblimersen sodium is targeted against Bcl-2, an important inhibitor of apoptosis. Bcl-2 has been shown to be overexpressed in a large number of tumours. Alicaforsen is also an important molecule of this type; it is a complementary antisense sequence to ICAM-1. Other phosphorothioate targets characterize cytomegalovirus and protein kinase C-alpha with Vitravene® (treatment of cytomegalovirus retinitis in AIDS patients) and Affinitac respectively (36, 38).

Second generation of ASOs

The second generation supports modifications at the 2'-position of the sugar moiety, for example the alkoxy substituent, confering stability against DNA or RNA cleaving enzymes. This increased stability is presented due to steric hindrance of nuclease attack at the phosphate group and absence of the 2' hydroxyl necessary for enzyme-substrate interaction. It was also established that an increased length of alkyl substitute reduced the base pairing properties; hence the methoxy-ethyl group is particularly suited. However, 2'-O-substituted oligonucleotides are unable to activate RNase H due to their RNA-like description. RNase H needs a DNA/RNA duplex to activate it. 'Gapmere' oligonucleotides were introduced to solve this problem. They have modified nucleotides at both the 3' and 5' end, but with an unmodified region in between. This provides good stability against exonucleases while the unmodified region is sufficient to activate RNase H activity (39, 40).

Third generation of ASOs and beyond

Introduction of a 6-aminoalkyl group at the 2'-position and others represents the third generation, zwitterionic ASOs. This modification improves stability against enzymatic degradation and also minimizes the electrostatic repulsion of the sense and antisense strands, leading to better hybridization affinity (41).

Other modifications include substitution of the polyanionic phosphate backbone by peptides (peptide nucleic acids, PNA). Changing the ribose moiety to morpholino groups (31) and introducing a methylene bridge between oxygen 2' and carbon 4' increase rigidity of the backbone (locked nucleic acids, LNA) (42, 43).

2.4.6. Conjugation of ASOs

After it was established that the first generation of ASOs, phosphorothioates, had good activity at target tissues via activation of RNase H activity and stability to exo- and endonucleases, other modifications have been made to develop activity, specificity and stability against these enzymes. One of the first approaches was the introduction of cationic groups to neutralize the electrostatic repulsion of the anionic backbone. This was done by conjugation of aminohexyl chains to carbon 5 of uridine. It was found that hybridization to the complementary DNA strand was increased. A subsequent investigation of the conjugation of aminohexyl showed that a reduction of the net anionic charge increased stability of the duplex and that this modification improved stability against nucleases. 2'-O-aminopropyl modified oligonucleotides have also been proven to increase nuclease stability compared to phosphorothioates (26, 34).

Currently research focus is on conjugation of ligands to oligonucleotides in order to support membrane penetration, such as lipophilic cationic lipids, liposomes and polymers. Complex forming structures of these ligands with ASOs allow a direct change in properties such as hydrophobicity, biodistribution or cellular uptake (34).

The melting temperature I of duplexes gives information about their stability: the higher the Tm, the greater the stability. Tm is the temperature at which half of the duplex has separated to its two single sense and antisense strands and half is still present as duplex. The addition of a single 5'-phospholipid resulted in a decrease in melting temperature compared to the unmodified ASO. However by placing a lipid at each 5'end the melting temperature actually increased. Additionally, lipid conjugated ASOs showed on 8-10 times higher cell association in comparison to unmodified ASOs. Another study has also shown that PNA attached to L-lysine residues (one, two or four) at the C-terminus had enhanced intracellular accumulation compared to unmodified PNAs (33). The extent of uptake is dependent on the number of conjugated lysine monomers. The lysine chain improves the transport through the cell membrane or enhances the release from endosomes (34).

So far the majority of peptides have been conjugated to the 3' or 5' end of the oligonucleotide and carbohydrate conjugation at the 2' position has been achieved via a 2'-succinyl linker (44). Conjugation of short lysine chains to the ASO oblimersen, targeted at bcl-2, has also been investigated. The results showed only minimal destabilizing effects due to the lysine modification (34). Other work has shown that polylysine conjugates of ligands for cell surface receptors such as transferrin have been used to prepare DNA complexes which have shown

greater cellular uptake efficiency (45). Hence it has repeatedly been shown that lysine modifications of ASOs are advantageous in enhancing cellular delivery. Similarly, other basic amino acids such as arginine and histidine could be used for ligand binding to oligonucleotides.

2.5. Alicaforsen

2.5.1. Introduction of alicaforsen (ISIS 2302)

Alicaforsen, also referred to as ISIS 2302, is a 20 base phosphorothioate oligodeoxynucleotide (sequence 5'GCCCAAGCTGGCATCCCTCA3') that inhibits ICAM-1 expression through an antisense mechanism (of action). ISIS 2302 is designed to specifically hybridize to a sequence in the 3'untranslated region of the human ICAM-1. ISIS 2302 selectively inhibits cytokine-induced ICAM-1 expression on a wide variety of human cells. In the body alicaforsen and other phosphorothioate ASOs are poorly absorbed after oral administration, but well absorbed after intraperitoneal, intradermal or subcutaneous injection. In blood, alicaforsen is bound to proteins in high extent. The compound does not cross an intact blood-brain barrier, but is otherwise widely distributed, principally in the liver, kidney and spleen. ISIS 2302 is metabolized as a native nucleic acid, by nuclease, primarily 3'exonuclease. This antisense inhibitor of ICAM-1 may be a promising drug for the treatment of Crohn's disease, rheumatoid arthritis, psoriasis and ulcerative colitis. Antisense technology offers potential efficient drug discovery, target specifity, and a wide therapeutical index in the treatment of a broad spectrum of human diseases (46, 47).

2.5.2. Alicaforsen in clinical trials

Phase 1

Therapeutical range in animal models (monkeys) was 2mg/kg. Therefore doses of alicaforsen from 0.06 to 2 mg/kg by 2 h intravenous infusion (i.v.) were selected as an appropriate target regimen for a phase 1 trial. All doses were well tolerated and dose-limiting toxicity was not observed. Pharmacokinetic behavior was very similar to that in monkeys; peak plasma levels were related to dose (10 μg/ml at 2 mg/kg) and the plasma distribution half-life was 60 min (48).

Alicaforsen was well tolerated subcutaneously in monkeys. In the phase 1 study, pharmacokinetic behavior was similar to that observed in monkeys, but dose-limiting toxicity was observed at 2 mg/kg every other day (46).

The maximum well tolerated dose was 0.5 mg/kg daily. Higher doses were associated with regional lymphadenopathy. All concentrations (from 50 to 200 mg/ml) of alicaforsen caused erythema and edema (46).

Phase 2

Phase 2 trails were conducted in four indications: Crohn's disease, rheumatoid arthritis, prophylaxis of acute renal allograft rejection and psoriasis.

The therapeutical range in mice was 2 mg/kg. With similar pharmacokinetics in mice and humans it was expected that this dose level would be therapeutic in humans as well. Therefore the phase 2 trials were designed from 0.5 mg/kg to 1 and 2 mg/kg every other day i.v. Trials were double-blinded, placebo-controlled and randomized (3:1 = study drug: placebo) and involved a 4-week treatment period. Treatment in the renal transplant study was for 2 weeks owing to logistical considerations and in addition this study was specified by two doses of 0.05 and 0.1 mg/kg. In each study, 17-52 patients were enrolled at one or two centers. Patients were followed for up to 6 months after the treatment period or until disease relapsed or failed to respond to study drug (46).

The study of Crohn's disease showed that alicaforsen produced clinical disease remissions. 13 of 22 patients (59%) achieved a response by a 70 point reduction in Crohn's Disease Activity Index (CDAI) score. 9 of 13 responders (41%) of the total patients in the trial experienced a clinical remission. Clinical remission was defined as a CDAI score of less than or equal to 150, with no increase in the use of corticosteroids or immunosuppressive, or need for surgery (37).

In the phase 2 of ulcerative colitis trial, alicaforsen produced disease improvement, as measured by changes in Disease Activity Index scores and other indicators of disease such as mucosal healing, decreases in rectal bleeding and in stool frequency. 13 of 22 patients (59%) achieved a response. Six months later at the end of the study, 10 of 13 (77%) patients continued to respond (37).

The rheumatoid arthritis trial was conducted by 43 patients. The best response was by 0.5 mg/kg, 40%. Further trials in rheumatoid arthritis with ICAM-1 antisense await the development of an oral formulation (49).

The only phase 2 open-labeled and uncontrolled psoriasis study had no evidence of a dose response. ODNs (oligodeoxynucleotides) do not distribute well in the direction to dermis, particularly epidermis with parental administration, but owing to molecular size and charge, it was thought that delivery via a topical formulation could not be readily achieved. However, a topical formulation has now been developed that achieves drug concentrations in the dermis and epidermis 2-3 logs higher than that achieved with i.v. dosing. Animal topical studies have been completed and a topical clinical trial in patients with psoriasis has begun (50).

Phase 3

In two identically designed phase 3 trials alicaforsen did not demonstrate statistically significant induction of clinical remissions compared to placebo. As a result of these data, the company Isis Pharmaceuticals has determined it will not invest further in the development of alicaforsen for Crohn's disease. Isis Pharmaceuticals is exploiting its expertise in RNA to discover and develop novel human drugs that bind to RNA. The company has 10 antisense products in development of clinical studies to treat metabolic, cardiovascular, inflammatory and viral diseases, and cancer. Unfortunately, alicaforsen was shown not to induce remissions in Crohn's disease in phase 3 trials, but Isis is committed to develop drug for ulcerative colitis (37). During the year 2007 Isis licensed alicaforsen to the company Atlantic Healthcare, to achieve together better results in pouchitis, ulcerative colitis and other inflammatory diseases.

2.6. RNA interfence (RNAi)

The discovery of RNAi goes back far as the year 1998. Many scientists were trying to understand the role of the gene expression in the nematode *Caenorhabditis elegans (C.elegans)*. In 1998 A.Z. Fire and C.C. Mello investigated the exogenous double-stranded RNA (dsRNA), which could induce potent and sequence-specific silencing of endogenous gene expression in *C.elegans*. They observed that double-stranded RNA causes effective and specific interference and is more efficient at producing interference than either strand individually. This observation has revolutionized study of gene function in biology, as a strategy for treating human diseases. On 2nd October 2006 A.Z. Fire and C.C. Mello were awarded the Nobel Prize in Physiology or Medicine for their discovery of RNAi – gene silencing of double-stranded RNA (51).

2.6.1. Mechanism and induction of RNAi

RNAi is a mechanism [see Figure 9], in which double stranded RNA can deplete protein biosynthesis in a sequence specific manner. This highly conserved natural antisense mechanism exists in most organisms.
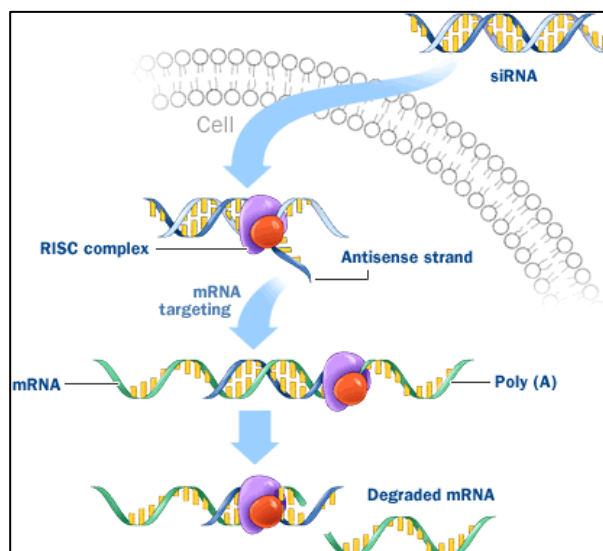
[Figure 9] RNAi activation. Adapted from Nastech Pharmaceutical Company Inc. (52).

Most ASOs induce degradation of the target mRNA by activating the endonuclease Rnase H in the nucleus. RNAi using double-stranded siRNA molecules occurs in the cytoplasm. RNAi is initiated by long stretches of duplex RNA, which undergoes processing into short 21-23 base-pair fragments (siRNAs) by a member of the RNAse-III family of nucleases called DICER. siRNA molecule contains 5' phosphate and 3' hydroxyl termini, and two overhanging nucleotides on their 3' ends (51, 53, 54).

siRNA consists of a sense and antisense strand, which binds to a multi-protein complex, called RNA induced silencing complex (RISC). In this form siRNA is inactive to conduct RNAi (54).

Therefore the next step of RNAi is followed by unwinding of the double stranded siRNA. The sense strand is removed for degradation by nucleases. RISC is transformed to its active form and the antisense strand of the siRNA links the complex complementarily by Watson-Crick base pairing to the target mRNA sequence (55).

Finally the target mRNA is degraded by the RISC nuclease Argonaute 2 thus prevented from being translated into a protein (56). The DICER step can be bypassed by using small siRNA molecules from the outset.

RNAi is related to different mechanisms [see Figure 10] comprising other small RNA sequences, known as microRNAs (miRNAs). These molecules regulate mRNA expression instead of regulating the inhibition of the translation into a protein. Substances miRNAs consist of 21-23 nucleotide pairs with 5' phosphate and 3'hydroxyl group, and have stem-loop structure distinguishing from siRNA. In cytosol single stranded miRNA sequences are converted from short hairpin RNA (shRNA) containing about 70 nucleotides (51, 57). Alternatively, shRNA molecules can be directly incorporated into the cell. One major disadvantage of long double-stranded RNA molecules exists: activation of the interferon system, thus leading to the induction of a cellular immune response (56).

miRNAs are processed by Dicer and incorporated into RISC. The miRNA-complex associates with ribosome and inhibits the translation of mRNA, which is complementary to the miRNA in its 3' untranslated region (51).

The first miRNA molecules, lin-4 and let-7, were discovered in *C. elegans*. Many miRNAs were identified in most multicellular organisms. Nevertheless, the function of most miRNAs including human, is not yet well defined. Further research is expected, because is believed miRNAs regulate one-third of human gene transcripts (or messenger RNAs) and are implicated in many human diseases. To manipulate miRNA levels represents an attractive approach for controlling gene expression (51).

RNA silencing represents an immune defence of genomes. RNAi system recongnizes the invading parasite, dsRNA, and amplifies the response to eliminate the foreign element. Virus infection of the cell forms double stranded RNA. The viral RNA is disrupted by RNAi, preventing the formation of new virus and protecting the cell from viral attacks. This anti-viral mechanism is presented in plants, worms and flies, whereas it is unclear how relevant it is for human (51).

RNAi secures genome stability by keeping transposons (mobile elements in genome) silent. If the mechanism is not efficient, the transposons are not kept under control, it causes deleterious effects in the genome (51).

In plants RNAi-like mechanisms keep chromatin condensed and suppress transcription. The phenomenon is not understood at the molecular level, however this action on chromatin is important for maintenance of genome integrity (51).
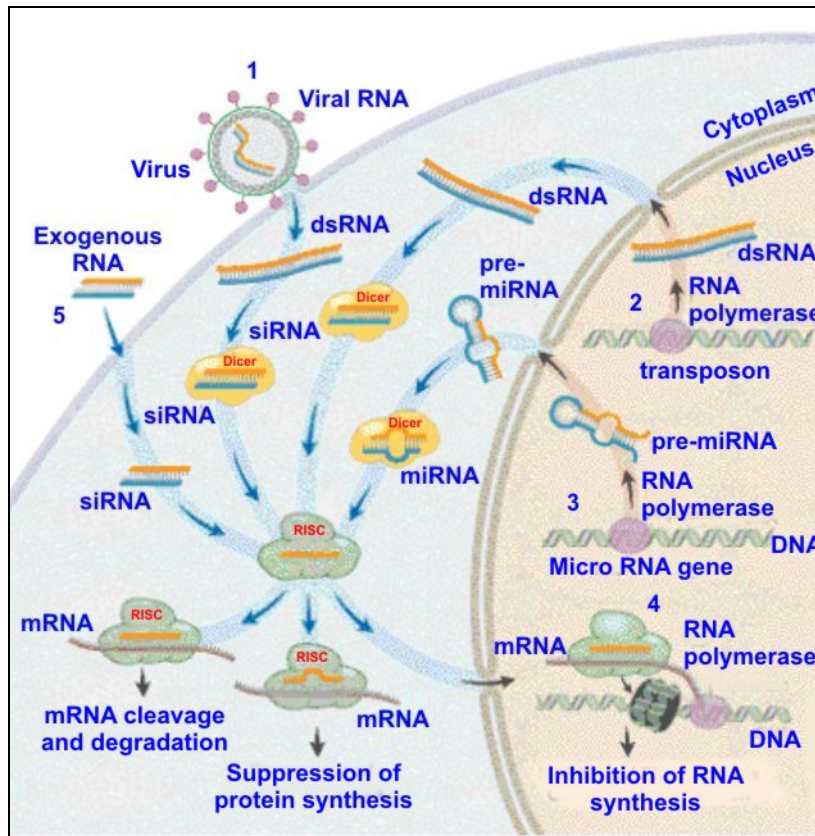
[Figure 10] Cellular processes dependent on the RNAi. RNAi protects the cell against viral infections [1], blocks the action of transposons [2], inhibits the protein synthesis due to miRNA generated within the cell [3], mediates RNAi-like mechanisms [4] and silences the activity of specific genes [5]. Adapted from www.nobelprize.org (58).

2.6.2. siRNA and ASOs

In summary RNAi, like ASOs, works in a sequence specific manner and results in suppression of the corresponding gene product. Synthetic siRNA and ASOs share some features, but there are also some differences between them [see Table 2]. Both antisense molecules inhibit synthesis of target proteins, but in different mechanism. The antisense strand of the siRNA directs the RISC to the target mRNA, complementary to the siRNA. An endonuclease within the RISC either degrades or inhibits translation of specific mRNA targets, depending on the degree of complementarity. Binding of ASOs to their target mRNA prevents protein translation by induction mRNA degradation by RNAse H or steric hindrance (55).

Both short nucleic acids are delivered to the target tissue with difficulties, both induce post-transcriptional gene silencing by targeting mRNA and cause mRNA cleavage (59).

In contrast to ASOs, siRNA is more difficult and expensive to prepare – two strands are needed, 2'-OH has to be protected for the synthesis and RNA is more difficult to handle. In cultured cells chemical modifications are less necessary for siRNA than for ASOs. Reduced needs for chemical modifications decreases toxicity to cells. However, siRNA is clearly not stable enough in vivo and in vitro, leading to the need for modifications in order to develop therapeutically active substances (59).

	ASOs	siRNA
Oligonucleotide chemistry	DNA or RNA	RNA
Structure	Single stranded, 16-30 nt	Double stranded, 19-21 nt with 2 nt overhangs
Intracellular processing from precursors	No	Yes, by Dicer
mRNA cleavage	Rase H	RISC
Standard doses effective *in vitro*	50-400 nmol/l	5-100 nmol/l
First *in vivo* use	1991	2002
Clinical trials	Numerous Phase 1-3 clinical trials ongoing; local or systemic administration	First studies initiated; so far only local administration

[Table 2] Comparison of ASOs and siRNA. Adapted from Cejka D, Losert D, and Wacheck V (59).

2.6.3. Modifications of siRNA

Chemical modifications of siRNA [see Figure 11] offer an approach to improve properties of these molecules. Duplex RNA can be quite stable in cell culture media with low concentrations of serum, in contrast to the situation *in vivo*, when double stranded RNA has less stability. Therefore is reasonable to develop chemical modifications, which might be effective in reducing non-specific effects, regulating distribution to target tissues and improving pharmacokinetic features of siRNA. Finally, optimizing delivery routes, including oral bioavailability, will be necessary to maximalize pharmaceutical opportunities of siRNA (59).

Some challenges to developing modifications of siRNAs, have been researched in past to improve the properties of nucleic acids (60):

- Various modifications (2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl and locked nucleic acid) enhance thermal stability of siRNA, which is characterized by melting temperature.

- Modifications situated near 3' or 5' termini of siRNA improve resistance to endonucleases (nucleases hydrolyzing 3' or 5' end of RNA), thus increase the stability of siRNA.

- Chemical modifications (PS, 4-thio, cholesterol) achieve tighter binding to serum proteins and prolonging half-life. This offers more opportunities for the siRNA to enter target tissues.

- Modifications reduce the complementarity of siRNA to non-target genes, thus side effects are minimized and specificity for target mRNA maximized.

- To be active to enter cells, siRNA must associate with the RISC. The mechanism for cellular uptake of siRNA and the structure optimization by chemical modifications is unknown.
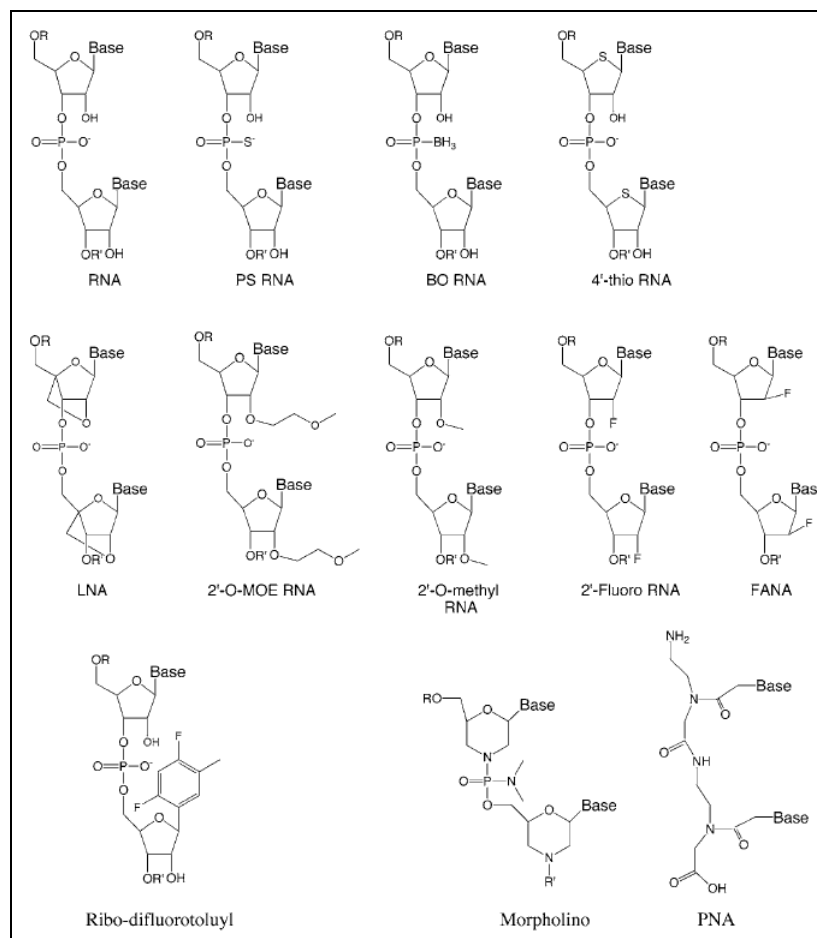
[Figure 11] Chemical modifications of siRNA. Adapted from Corey DR (60).

Phosphorothioate linkages

The phosphorothioate linkage is created by replacing the non-bridging oxygen in the phosphodiester backbone with a sulphur atom. Probably phosphorothioate linkages have been introduced as the most important modifications for ASOs. Phosphorothioates enhance

nuclease resistance and binding to serum proteins. Even the stability and the half-life period of oligonucleotides is improved, its usage is accompanied by *in vivo* toxicity, when the substitute linkage is placed at alternating positions (60, 61). Only one or two terminal phosphorothioate modifications are suggested (62).

Though duplex RNA is quite stable, the supplement of phosphorothioate into siRNA provides the improvement in serum stability and tissue accumulation. Nevertheless, the effect of the phosphorothioate linkage on siRNA *in vivo* remains to be determined (59).

Boranophosphate linkages

The boranophosphate linkage contains a boron atom in place of the non-bridging oxygen atom on the backbone between two ribonucleotides. This modification has been studied relatively shortly, but reports stated improved gene silencing at lower concentration than native or phosphorothioate siRNAs (77). Chemical methods for introducing the boron modification are needed to be optimized (63).

Locked nucleic acid (LNA)

LNA consists of a methylene bridge between 2' and 4' carbons of the ribose moiety. The linkage locks the ribose ring into a conformation formed by RNA after hybridization and the affinity of LNA oligonucleotides to complementary sequences is enhanced. The LNA modification improves the thermal stability of RNA duplexes and stabilizes them against degradation by nucleases (59). ASOs containing LNA bases have been showed high effectivity, but also liver toxicity in mouse models (64).

2'-modified RNA

Many siRNA analogs include substitutions for the hydroxyl group on the 2' carbon atom of the ribose moiety. These analogs include 2'-O-methyl RNA, 2'-O-methoxyethyl (2'-MOE) RNA and 2'-fluoro RNA. These modifications improve the affinity of the siRNA to complementary mRNA and increase its resistance to nucleases. The 2'-O-methyl and 2'-MOE antisense nucleotides have been tested in clinical trials (60).

siRNA oligonucleotides with alternating 2' modifications (2'-O-methyl and 2'-fluoro) show higher rate of silencing gene expression in human cells compared to the native siRNA (65). Incorporation of 2'-O-methyl nucleotides near the terminus of the siRNA strand complementary to the mRNA target can reduce side effects (66).

Another group of 2' modifications is 2'-deoxy-2'-fluoro-β-D-arabinonucleic acid (FANA). FANA nucleotides are based on the arabinose sugar instead of the ribose and have similar stereochemistry at 2'-position to 2'-O-methyl and 2'-fluoro RNA. FANA substitutions are tolerated for gene silencing in positions 3' and 5' of the strand (67).

4'-thio modified RNA

Thio modified oligonucleotides consist of a sulphur atom in place of the oxygen attached to the 4' carbon of the ribose moiety. This siRNA modification improves resistance to nuclease and binding to serum proteins. Combining 4'-thio bases with 2' modifications such as 2'-O-methyl or 2'-MOE improve other properties of siRNA (60, 68).

Ribo-difluorotoluyl nucleotide

Ribo-difluorotoluyl nucleotides modify neither the ribose moiety nor the phosphate backbone, but the ribonucleotide base. These modifications are an interesting group, because they cannot form usual Watson-Crick base-pairs (69).

Uncharged nucleic acid mimics

Uncharged nucleic acid mimics, such as peptide nucleic acids (PNA) and morpholino monomers differ from other modified RNA by chemical properties. Despite of their chemical difference they can be attached to RNA strands and form novel oligonucleotides with different properties. One of their main disadvantages is a slow application, because obtaining modified siRNA is not a straightforward process. Since the usual methods for RNA synthesis are not compatible (60).

2.6.4. Conjugations of siRNA

Another improvement in the potency of siRNA, such as binding to proteins or cellular uptake, can be achieved by conjugation of small molecules and peptides to siRNA duplex. Enhanced binding to serum proteins might prolong biological half-life and tissue distribution. Again, these tasks have been intensively explored by ASOs, and are now being applied to duplex RNA. siRNA conjugates are concepted to provide specificity for the mRNA target, improve biodistribution, cellular uptake and other properties. A weakness of conjugation is presented by building even larger and more complex siRNA (60).

A conjugate of cholesterol has been tested to inhibit the expression of APOB in mice. Gene silencing of APOB by cholesterol-RNA duplex conjugate was showed effectively (62).

siRNA conjugated to peptides and polyethylene glycol reported gene inhibition in cultured cells. (70, 71) Also conjugation to aptamers (small structured nucleic acids selected to bind target proteins) showed blocking expression in cell cultures (72).

It might be possible to combine siRNA with the specificity of antibodies such as protamin antibody fusion protein, which was introduced in one study (73). The antibody bound to HIV-1 envelope protein. The conjugate produced gene expression inside cells. Results from testing on animals were less satisfactory; therefore more research will be needed.

2.6.5. Another improvement of siRNA

Additional strategy for clinical development of siRNA, besides modifications of siRNA oligonucleotides, is to increase their efficiency by mixing RNA with liposomes, cationic lipids, nanoparticles or polyethylenimine that facilitate uptake by cells and tissues resisting entry of siRNA. These compounds improve protection of the RNA against the nucleases (56, 60).

Other possible way for gene delivery is the use of viral vectors. The successful strategy of application the retroviral, lentiviral, adenoviral and adeno-associated vectors have been reported. Although viral vectors provide advantages in transfection efficiency, potency and longevity, they need more laborious studies to exceed occurred obstacles such as oncogenic mutagenesis (55).

Another approach to the task of biodistribution is to facilitate the problem by concentrating on local distribution more than systematic. Local delivery acts in smaller, more protected area within the body, and lower doses of siRNA are sufficient. Local administration simplifies distribution, decreases side effects and reduces the amount and cost of siRNA dose. The first clinical trials with intraocular application of an injected vascular endothelial growth factor (VEGF)-specific siRNA assessed as a possible treatment for macular degeneration. Inhalation of siRNA qualified as a feature treatment for infection caused by respiratory syncytial virus (60). Considering the systemic application, some studies rely on hydrodynamic transfection of siRNA (rapid high pressure injection of large volume of solution with siRNA). Nevertheless, side effects have been observed on animals and using this method on humans is unacceptable (56).

40

Although chemical modifications and conjugations might be less necessary for local distribution than systematic, it is certain that the modifications can improve the properties of potential drugs.

2.6.6. siRNA in clinical trials

Since the discovery of the role of siRNA molecules inducing RNAi, the direct application of siRNAs has been investigated in vitro and in vivo. In vitro, several transfection reagents help the delivery of siRNAs into cells. In vivo application of siRNAs requires the development of more strategies to enhance the delivery of siRNAs into specific target tissues and diminish the side-effects and toxicity in the target (56). Intense research and clinical trials [see Table 3] are being aimed at developing siRNAs for therapeutical purposes.

In 2004, Acuity Pharmaceuticals initiated the first clinical trial on humans with siRNA molecule Cand-5 (bevasiranib). Bevasiranib is supposed to inhibit the production of VEGF, which has a central role in the ocular neovascularization and vision loss in age-related macular degeneration (AMD) and diabetic retinopathy. Currently bevasiranib proceeds in the phase 3 clinical trial by Opko Health Company and is believed that bevasiranib could treat the blindness of patients with AMD (51, 55, 75).

Independently in 2004, Sirna Therapeutics started a clinical trial with siRNA (Sirna-027) targeting VEGF receptor 1 for AMD. Sirna-027 completed successfully a phase 1 of clinical trial. In 2006 Sirna Therapeutics together with its strategic partner Allergan Company has entered a Phase II with Sirna-027 for the treatment of AMD (51, 55, 75).

Further clinical trials for siRNA-bases are intended in various diseases, including cancer, viral infections, neurodegenerative and metabolic disorders. Alnylam Pharmaceuticals, the Sirna company, has initiated clinical trials for targeting respiratory syncytial virus (RSV). A clinical trial is investigating local administration of ALN-RSV01 by inhalation. The inhaled formulation of ALN-RSV01 used in the Phase I study was delivered via a nebulizer and an intranasal spray. The company completed the Phase I clinical trial of ALN-RSV01 using an intranasal formulation and entered the Phase II clinical trial (55, 56).

DRUG (MANUFACTURER)	TYPE OF COMPOUND	INDICATION (MOLECULAR TARGET)
PHASE 3		
Genansense/Oblimersen (Genta)	Anti-sense oligonucleotides	Varied cancer (bcl-2)
Cand-5/Bevasiranib (Opko)	siRNA	Macular degeneration (VEGF)
PHASE 2		
ISIS 2302/alicaforsen(Isis)	Anti-sense oligonucleotides	Ulcerative colitis (ICAM1)
ISIS 301012 (Isis)	Anti-sense oligonucleotides	High cholesterol (apoB-100)
ISIS 113715 (Isis)	Anti-sense oligonucleotides	Diabetes (PTP1B)
ISIS 104838 (Isis)	Anti-sense oligonucleotides	Rheumatoid arthritis (TNF-alpha)
ATL1102 (ATL/Isis)	Anti-sense oligonucleotides	Multiple sclerosis (VLA4)
OGX-011 (OncoGenex/Isis)	Anti-sense oligonucleotides	Prostate cancor (clusterin)
SPC2996 (Santaris)	Anti-sense oligonucleotides	B-cell lymphoma (BCL2)
Resten-NG (AVI)	Anti-sense oligonucleotides	Restenosis (MYC)
Sirna-027 (Sirna)	siRNA	Macular degeneration (VEGF)
AVI-5126 (AVI)	Anti-sense oligonucleotides	Cardiovascular disease (c-myc)
ASM8 (Topigen)	siRNA	Asthma (CCR3 and IL-5)
PHASE 1		
LY21181308 (Lilly/Isis)	Anti-sense oligonucleotides	Cancer (survivin)
LY2275796 (Lilly/Isis)	Anti-sense oligonucleotides	Cancer (eIF4E)
GRN163L (Geron)	Anti-sense oligonucleotides	Cancer (telomerase)
AVI-4065 (AVI)	Anti-sense oligonucleotides	Hepatitis C (HCV virus)
AVI-4557 (AVI)	Anti-sense oligonucleotides	Drug metabolism (cytochrome P)
LErafAON-ETU (Neopharm)	Anti-sense oligonucleotides	Cancer (c-raf)
AVI-4658 (Imperial college London)	Anti-sense oligonucleotides	Muscular dystrophy (exon 51)
EZN-2968 (Enzon)	Anti-sense oligonucleotides	Cancer (anti-HIF-1α)
GTI-2040 (Lorus)	Anti-sense oligonucleotides	Cancer (R2 component of ribonucleotide reduktase)
AEG35156 (Aegera)	Anti-sense oligonucleotides	Cancer (X-Linked Inhibitor of Apoptosis = XIAP)
ALN-RSV01 (Alnylam)	siRNA	RSV infection (RSV virus)

[Table 3] siRNA versus antisense oligonucleotide based therapeutics in clinical trials. Adapted from Corey DR (77), the database of clinical trials developed by U.S. National Institutes of Health (NIH), through its National Library of Medicine (NLM) (78) and www.asuragen.com (79).

There are many good reasons for developing siRNA compounds as therapeutic drugs. One of the main advantages of siRNA technology is the option to synthesize a specific siRNA compound for any target gene. This flexibility provides a clear advantage compared with small molecules based drugs. However even the most careful synthesis does not guarantee exclusive target specificity. Off-target effects might be a major challenge for the intended selective silencing of the target gene. According to clinical trials initiated so far, show limitation to achieving gene silencing by local delivery of siRNA. For successful systematic administration, chemical modifications as well as lipid based formulation in combination with specific conjugates for cell targeted uptake of siRNA might break the way for siRNA based therapy (55).

2.6.7. siRNA in prospective therapy

Many human diseases are caused by inappropriate endogenous or exogenous gene expression. Actually drug therapy is based only on about 500 molecular targets, which can be influenced by small molecules. Potentially every gene would become compliant to regulation with siRNA, thus new opportunities can arise in the drug development. Recent studies are focused on gene silencing of a target gene by siRNA [see Table 4] as potential therapeutics (55).

siRNA in cancer

The inhibition of tumor growth is the primary goal of the cancer treatment. Target molecules present genes shown previously to be relevant for tumor growth. Studies involve genes such as *Bcl-2*, *p53* and *k-ras* (51).

Several diseases, such as cancer, are characterized by the uncontrolled growth of blood vessels. This might result from the over-expression of pathogenic genes. One strategy to overcome this problem is using a combination of multiple drugs. The combination of siRNAs targeting VEGF-A, VEGFR1 and VEGFR2 has demonstrated improved anti-angiogenesis potency compared with siRNAs targeting only one factor (80). Combining multiple siRNAs targeting angiogenic factors may enable the identification of potent anti-angiogenic agents for potential therapeutic applications. Hence, there are some attractive siRNA targets available in the struggle against cancer and angiogenesis (51).

siRNA in neurological diseases

The siRNA mediated gene silencing of β-secretase (BACE1), which is up-regulated in the brain of patients with Alzheimer's disease (AD). The knockdown of BACE1 decreased the secretion of β-amyloid peptides Ab140 and Ab142 in mouse neurons, which are designed to express amyloid precursor protein (APP) and also decrease the peptides in neurons carrying two human APP mutations (KM670/671NL). This discovery is promising, thus making BACE1 a potential target for siRNA therapeutics for AD in future (81).

siRNA in liver diseases

Several studies demonstrate that siRNA therapeutics could be beneficial in treating liver disorders. In the mouse liver investigation has found that expression of *Fas* (*Fas* mediated apoptosis is implicated in many hepatic disorders) was inhibited in mouse hepatocytes, indicating that siRNAs are stable and prevent acute hepatitis (82).

siRNA in viral diseases

Various studies focus on antiviral targets for RNAi. Using hepatitis C viral (HCV) protein with luciferase showed that they can inhibit the expression of the HCV protein with specific siRNAs (83). Gene silencing of HIV and polio viral propagation using siRNA might make the treatment of viral infections by RNAi vectors possible. (84) siRNAs were applied during an attempt to achieve stronger anti-severe acute respiratory syndrom corona virus activity, with remarkable success (85).

siRNA in sclerosis

Recent reports offer a new therapy for dominant gene disorders, such as familial amyotrophic lateral sclerosis (ALS). ALS is characterized by motor neuron degeneration, paralysis and death. Mutations in Cu/Zn superoxide dismutase in ALS causes the death of motor neurons, a process that is incompletely understood. Studies have shown that siRNA knockdown of gene expression of superoxide dismutase can delay disease and death in mice. Nevertheless, the major obstacle is to successfully differentiate and degrade mutant RNAs leaving wild-type transcripts unaffected (86).

CATEGORY	siRNA TARGET
CANCER	
Breast cancer	Bcl-2, VEGFR1 and VEGFR2, CXCR4
Cervical carcinoma	E6, E7 (HPV)
Colon cancer	MMP-9
Gastric cancer	Bcl-2, VEGFR1 and VEGFR2
Leukaemia	C-raf and bcl-2
Ovarian cancer	VEGF
Prostate cancer	P110α and p110β of PIP3 kinase
Small cell lung carcinoma	Skp-2
ANGIOGENESIS	
Ocular neovascularization	VEGF, VEGFR1 and VEGFR2
Rheumatoid arthritis	Akt, GG2-1, ASC
Tumor angiogenesis	VEGF
VIRAL DISEASES	
Influenza A	NP, PA, PB1, PB2, M, NS
Hepatitis B	Core region RNA
Hepatitis C	EMCV-IRE5, NS3, NS5B, NA
HIV1	Gag, pol, CCR5,CD4, nef, gp120
Poliovirus	Capsid, airal polymerace

[Table 4] Some siRNA targets in cancer, angiogenesis and viral diseases. Adapted from Pushparaj PN and Melendez AJ (51) and Hadj-Slimane R, Lepelletier Y, Lopez N, Garbay C, and Raynaud F (87).

Explanation of receptor abbreviations targeted by siRNA in human body: HPV (human papillomavirus); PIP3 (phosphatidylinositol 3,4,5-trisphosphate); VEGFR (VEGF receptor); ASC (antistratum corneum); NP (nucleocapsid protein); PA (a component of the RNA transcriptase); PB1, PB2 (viral polymerase B1 and B2); M (matrix); NS (non-structural protein); EMCV-IRE5 (encephalomyocarditis virus-internal ribosome entry site); NS3, NS5B (non-structural protein 3 and 5B); NA (neuraminidase)

2.7. Cell culture

2.7.1. Human Umbilical Vein Endothelial Cells (HUVEC)

The endothelial tissue is highly active and closely involved in numerous physiological processes. If the function of the endothelial cells is impaired, the consequences can be numerous diseases such as atherosclerosis and cancer. Biological techniques for the

characterization of the endothelium have improved; therefore today it is possible to determine the extensive heterogeneity of endothelial cells.

HUVEC are isolated from human umbilical vein. They are cryopreserved at the end of primary culture and can be cultured and propagated at least for 6 population doublings. HUVEC are responsive to cytokine stimulation in case of the expression of cell adhesion molecules. These cell systems are commonly used for physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, and fibrinolysis (88).

The isolation and culture of these cells have several disadvantages, including the risk of infection, exogenous growth factor requirement, and low proliferative capacity. The main advantage of HUVEC culture is the wide availability of umbilical cords (89).

2.7.2. Endothelial Cell Line (ECV 304 cells)

Early reports indicated that ECV304 cells were spontaneously immortalized and transformed cell line derived from a Japanese HUVEC culture. Many morphological, immunochemical, and genetic studies provided further evidence that ECV304 cells were a valuable biomedical research tool and have been used for studies of angiogenesis, cell migration, cytokine expression, or signal transduction of vascular endothelial growth factor (90).

Endothelial cells separate the intra- and extravascular space and regulate transport processes among these compartments. ECV304 cells display further characteristics of endothelial cells, including expression of thrombomodulin, receptor CD51, secretion of plasminogen activator inhibitor I and endothelia. ECV304 cells also express proteins characteristically found in epithelial cells, including E-catherin and desmosomal proteins desmoplakin, desmocollin and desmoglein. In conclusion, ECV304 cells express many endothelial markers and form specialized intercellular junctions that have some epithelial features. Thus this reportedly endothelial derived human cell line may be differentiated toward an epithelial phenotype (91). However, this permanent cell line offers several advantages over primary cultured endothelial cells, including uniform batches, which allow valid comparisons between results from different laboratories (91). These cells are relatively easy to maintain and can undergo multiple passages while retaining the same phenotype and have similar behavior to primary brain endothelial cultures (92). For these reasons ECV304 cells were used in this project.

2.8.   Aim of the work

Critical parameters of unmodified ODNs and siRNA are rapid enzymatic degradation, poor stability and inability to cross cell membranes. Currently a lot of research work is done in the field of modifications in order to overcome these problems.

As previous results have shown the 2'-lysine modification can be advantageous in aiding cell membrane penetration. Third generation of ASOs with zwitterionic introduced 6-aminohexyl group at the 2'-position to increase stability against enyzymatic degradation, minimize the electrostatic repulsion of the sense and antisense strands leading to improved hybridization affinity. It remains uncertain if a 2'-O-lysylaminohexyl modification shows a higher affinity for gene silencing than unmodified molecules of oligonucleotides. The ICAM-1 target is the model used to evaluate the influence of this modification.

Aim of this work is to examine the effectivity, of 2'-O-lysylaminohexyl modifications on DNA and siRNA. Previous work showed that 2'-O-lysylaminohexyl modification influence stability against enzymatic degradation, penetration into the cell and lead to a reduction of net charge, which should have favorable effect on duplex stability.

Efficiency of modifications in suppressing target protein expression is tested with the sequence of alicaforsen. Alicaforsen is a phosphorothioate targeting ICAM-1, which is currently in clinical phase 3 against ulcerative colitis. It has been described to successfully down-regulate ICAM-1 in many cell culture models, but has not been examined on a BBB cell line yet. In this study the ECV304 cell line is used as in vitro model for the test of alicaforsen, alicaforsen analog (the same sequence, but without the sulphur atom in the backbone), alicaforsen with one, two and three modifications (1U*, 2U*, 3U*) and a siRNA sequence targeted against ICAM-1 either, also with one, two and three modifications (1U*, 2U* and 2U*).

The 2'-O-lysylaminohexyl is linked to the uridine base, which replace one, two or three thymine bases. The 2'-O-lysylaminohexyl modification is done in three repeats (1U*, 2U* and 3U*) on DNA and siRNA sequences, respectively. The main focus of this study is to demonstrate if the 2'-O-lysylaminohexyl is an efficient modification to stabilize oligonucleotides and siRNAs within the cell, which would also be reflected in a higher inhibition of the target protein. Possibilities of modification intended to examine are in the middle and/or at the end of the molecule. Does the location of the 2'-O-lysylaminohexyl modification have an effect on the inhibition? Is the in vitro effect correlated with the number

of modifications? Which DNA and siRNA modification is the most effective in gene silencing?

Is it possible to predict the most effective concentration among modified and unmodified DNA and siRNA sequences?

3. MATERIALS

3.1. Apparatus

- Biofuge Stratos Centrifuge (HERAEUS Instruments, Hanau, Germany)
- BIOSAFE 3 Laminar air flow workbench (EHRET, Emmendingen, Germany)
- Cell tissue flask 25 and 75cm^2 (Creiner Bio-one GmbH, Frickenhause, Germany)
- CertoClav Sterilizer GmbH (Traun, Austria)
- DNA synthetizer (PolyGen, Langen, Germany)
- Driver machina (HERAEUS Instruments, Hanau, Germany)
- EAZY Breeze Drying Frame (HOEFER Scientific Instruments, San Francisco, USA)
- EAZY Breeze Gel Dryer (HOEFER Scientific Instruments, San Francisco, USA)
- Electrophoresis equipment (Bio-Rad, Hercules, USA)
- Film cassette and films (Amersham Biosciences, Freiburg, Germany)
- GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, USA)
- MR 3001 K Kontaktthermometer 3mA (Heidolph, Germany)
- Multiwell^{TM} 6well (Becton Dickinson Labware, NJ, USA)
- NAPCO 600O Water-Jacketed CO_2 Incubator (NAPCO, NY, USA)
- Orion 250A Ph-meter (Orion, Boston, USA)
- Power Pac 100 (Bio-Rad, Hercules, USA)
- RET Kontaktthermometer (IKA, Staufen, Germany)
- Sartorius weights (Sartorius, Göttingen, Germany)
- Shaker 3016 (GFL, Burgwedel, Germany)
- Thermomixer Comfort (Eppendorf, Hamburg, Germany)
- Trans-blot SO Semi-dry transfer cells (Bio-Rad, Hercules, USA)
- U-1700 Spectrophotometer (Pharma Spec, Shimadzu, China)
- VORTEX-2-Genie (Scientific industries, NY, USA)

3.2. Chemicals and reagents

- Acetonitrile 30% (J.T. Baker B.V., Deventer, Holland)
- Acrylamide 2X, research grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- APS (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- Aqua purificata (Elisabeth Schubert, Gesselschaft GmbH, Vienna, Austria)
- Amonium acetate (SIGMA, Steinheim, Germany)
- Ammonium persulphate, analytical grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- Amphotericin B (SIGMA, Steinheim, Germany)
- Bromophenolblue Na-salt, research grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- Bovine serum albumin (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- n-Butanol, for synthesis (MERCK, Darmstadt, Germany)
- CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), research grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- Coomassie Brilliant Blue G 250, pure (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- Dithiothreitol, research grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- D-MEM + 4500mg/l Glucose + L-Glutamine + Pyruvate (GIBCO, Invitrogen, Paisley, UK)
- Dodecylsulphate Na-salt, for biochemical application (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- EDTA (ethylene diamine tetraacetic acid)-disodium, analytical grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)
- DPBS 10x (GIBCO, Invitrogen, Paisley, UK)
- Ethanol 96% (Fischer scientific, Loughborough, UK)
- Ethanol absolute, Aethanolum absolutum ÖAB (NYCOMED Austria GmbH, Linz, Austria)
- F12 Nutrient mixture (Ham) 1x (GIBCO, Invitrogen, Paisley, UK)
- Formaldehyde solution 36,5%, puriss.p.a. (Fluka Chemie GmbH, Buchs, Switzerland)
- Gelatin (SIGMA, Steinheim, Germany)

- Glycine, analytical grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)

- HBSS (GIBCO, Invitrogen, Paisley, UK)

- Heparin (MP Biomedicals Inc., OH, USA)

- HEPES, 1mol/l (GIBCO, Invitrogen, Paisley, UK)

- Hydrochloracid, 1mol/l (MERCK, Darmstadt, Germany)

- Isopropanol (ACROS ORGANICS, New Jersey, USA)

- IMDM 1x + L-Glutamine + 25mM HEPES (GIBCO, Invitrogen, Paisley, UK)

- L-Glutamine 200mM(SIGMA, Steinheim, Germany)

- Lipofectamine 2000 Reagent (Nitrogen, Paisley, UK)

- Methanol, „Baker analyzed" (J.T. Baker B.V., Deventer, Holland)

- Milk powder, blotting grade (Carl Roth GmbH, Karlsruhe, Germany)

- Milli-Q grade water (Millipore Corporation, Bedford, MA, USA)

- MW marker (Bio-Rad, Hercules, USA)

- N,N´-methylenebisacrylamide 2X, research grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)

- N,N,N',N'-tetramethylethylene diamine, research grade(SERVA Electrophoresis GmbH, Heidelberg, Germany)

- Natrium chlorid Ph.Eur. (HESTAG, Vienna, Austria)

- Newborn Calf Serum Heat Inactivated Origin New Zeland (GIBCO, Invitrogen, Paisley, UK)

- Optimem (GIBCO, Invitrogen, Paisley, UK)

- Ortho-phosphoric acid 85% (FLUKA, Neu Ulm, Germany)

- Penicilin/streptomycin (GIBCO, Invitrogen, Paisley, UK)

- Phosphoric acid, „Baker analyzed"(J.T. Baker B.V., Deventer, Holland)

- Precision plus protein, all blue standards (Bio-Rad, Hercules, USA)

- Tetrabutyl ammonium fluoride (SIGMA, Steinheim, Germany)

- Tetrahydrofuran (SIGMA, Steinheim, Germany)

- Thiourea, puriss. p.a. (Fluka Chemie GmbH, Buchs, Switzerland)

- Transferrin (SIGMA, Steinheim, Germany)

- Triethyl ammonium acetate (SIGMA, Steinheim, Germany)

- Trifluoro acetin acid (MERCK, Darmstadt, Germany)

- Tris (trishydroxymethylamino methane), research grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)

- Triton X 100 (SERVA Electrophoresis GmbH, Heidelberg, Germany)

- Tween, pure (SERVA Electrophoresis GmbH, Heidelberg, Germany)

- Trypsin, Sequencing Grade (Roche Diagnostics GmbH, Manheim, Germany)

- Urea, analytical grade (SERVA Electrophoresis GmbH, Heidelberg, Germany)

3.3. Solutions

- *Acrylamide (AC) stock solution (30.8%)*

	400 ml
Acrylamide 2x 30%	120 g
N, N´-methylenebisacrylamide 2x 0.8%	3.2 g
Ad 400 aqua purificata	276.8 g

Water is weighted out extra, given to AC and stirred well. Stored by 4°C.

- *Acrylamide stock solution (40%)*

	400 ml
Acrylamide 2x 40%	132 g
N, N´-methylenebisacrylamide 2x 0.8%	3.2 g
Ad 400 aqua purificata	264.8 g

Water is weighted out extra, given to AC and stirred well. Stored by 4°C.

- *Ammoniumpersulphate 10%*

	50 ml
Ammoniumpersulphate 10%	5 g

1 ml aliquots

Stored by -20°C.

- *Anode buffer 10x*

	1000 ml
Tris 25mM	30.2 g
SDS 0.1%	10 g

Stored by room temperature.

- *Blocking solution*

5% BSA or milk powder in 0.1% TBST

5 g milk powder in 100 ml of 0.1% TBST, well stirred.

TBST: 100 ml TBS, 1 ml (0.1%) Tween ad 1000 ml water.

- *Blotting buffer*

	1000 ml
Tris 25mM	3.3 g
Glycin 192mM	14.4 g
Methanol 20%	200 ml
	ad 1000 ml water

Stored by 4°C.

Recoverable!

- *Bradford reagent*

	500 ml
Coomassie brilliant blue G250 0.01%	50 mg
Ethanol 5%	25 ml
Phosphoric acid 10%	50 ml

Firstly ethanol and acid are stirred for 15 minutes. Then is added water and all substances are stirred for 30 minutes. Finally Bradford reagent is filtered through a folded filter and stored by 4°C.

- *Buffer for primary and secondary antibodies = 1% milk powder*

	100 ml
Milk powder	1 g
0.1% TBST	ad 100 ml

- *Cathode buffer 10x*

	1000 ml
Tris 25mM	30.2 g
SDS 0.1%	10 g
Glycin 192mM	144.2 g

Stored by room temperature.

- *Developing solution*

Mix together ECL plus 3 ml A + 75 μl B pro 1 gel.

- *Gelatine 1%*

	400 ml
Gelatine	4 g
Aqua purificata	ad 400 ml

Gelatine is stirred by 100°C. It is weighted until gelatine is melted (half hour). It is sterilized in the autoclave.

- *IMDM+F12 1:1/22.5% Newborn calf serum (NBS)*

End volume of medium in six-well plate is 1.5 ml; because 0.5 ml of IMDM (Iscove's Modified Dulbecco's Media) + F12 1:1/22.5% NBS medium is given on 1ml of solution in 6-well plate. Hence is given one third and triple compound of NBS is used. The dilution from it is 1:3 = 7.5% * 3 = 22.5% of NBS (7.5% is the normal serum concentration in the medium of ECV304).

The rest medium is IMDM and Ham's F12 = 1:1.

	30 ml
NBS	6.75 ml [30 ml=100%; 22.5%= x ml]
IMDM	11.63 ml [(30 ml-x)/2]
Ham's F12	11.63 ml

Solution is through syringe and syringe filter (0.22 μm) sterile filter.

- *Laemmli buffer*

	50 ml
Tris 250mM	1.514 g
SDS 8%	10 ml
Glycerol 20%	1 ml
Bromophenolblue 0.02%	33.5 ml

1 ml aliquots

Stored by room temperature.

For application is given 6.2 mg/ml.

Stock solution of bromophenolblue 1% = 0.1 g/10 ml; stored by 4°C.

- *Lysis buffer*

	50 ml
Urea 8M	24 g
Thiourea 2M	7.6 g
Triton X 100 0.5%	250 μl
CHAPS 2%	1 g
EDTA 5mM	0.093 g
Aqua purificata	3.41 g/ml

500 μl aliquots

Stored by -20°C.

For application immediately before lysis is added:

DTT	5 mg/ml
Protease Inhibitor Cocktail	10 μl/ml

- *PBMEC-FIB without fibronectin*

	500 ml
Ham's F12 (Ham's F12: IDMD = 1:1)	104 ml
IMDM = 1:1	104 ml
7mM L (+) glutamine	17.5 ml (200mM stock solution)
5 μg/ml transferrin	250 μl
5 μg/ml heparin	250 μl (1000 U/ml = 10 mg/ml)
3.75 vol. % newborn calf serum (NBS)	19 ml
1 vol. % penicillin/streptomycin 10^4U/ml	5 ml
ACM = C6 (astrocytes cond. medium)	250 ml
Amphotericin B	500 μl

PBMEC C6 cell medium is removed every second day from a confluent culture of C6 cells (to gain growth factors for endothelial growth comes in vivo from astrocytes) and is mixed 1:1 with fresh PBMEC medium.

If is used PBMEC without fibronectin, gelatine is sufficient.

- *Separating gel buffer 4x*

	500 ml
Tris 1.5M	90.8 g
SDS 0.4%	2 g

Set up with 6 N HCl to pH 8.8

Stored by room temperature.

- *Stacking gel buffer 4x*

	100 ml
Tris 0.5M	6.06 g
SDS 0.4%	0.4 g

Set up with 6 N HCl to pH 6.8

Stored by room temperature.

- ***TBE 10x solution (Tris borat EDTA buffer)***

	1000 ml
Tris	108 g
Bor acid	55 g
0.5 M EDTA	40 ml
	Ad 1000 ml aqua purificata

Preparation of 0.5M EDTA solution:

	100 ml
	18.615 g EDTA
	ca. 60 ml aqua purificata

Set up with 2 N NaOH to pH 8, then ad 100 ml aqua purificata.

- ***TBS solution1x***

	1000ml
Tris 0.5M	30.12 g
Natrium chloride	84 g
Aqua purificata	ad 1000 ml

Set up to pH 7.8.

- ***Trypsin/EDTA***

	200 ml
Trypsin/EDTA	20 ml
HBSS (10)	20 ml
PBS without Ca^{2+} and Mg^{2+}	154 ml
HEPES 1M	2 ml
$NaHCO_3$, 7.5% vol.	2 ml
Penicillin/streptomycin, 10^4	2 ml

Media are sterile filtered in sterilcups.

4.   METHODS

4.1. Synthesis

Phosphordiester, triester and H-Phosphonate methods for the synthesis of ASOs have been replaced in most cases by the phosphoroamidite method. The phosphoroamidite method is built on the synthesis of the phoshorothioate backbone (26).

In this project the following DNA and siRNA sequences were synthesized on a PolyGen DNA-Synthesizer according to this method [see Table 5].

Alicaforsen	GCC-CAA-GCT-GGC-ATC-CGT-CA	PS
Alicaforsen control	GAC-GCA-TCG-CGC-CTA-CAT-CA	PS
Alicaforsen sense	TAG-CGG-ATG-CCA-GCT-TGG-GC	PS
Alicaforsen analog	GCC-CAA-GCT-GGC-ATC-CGT-CA	PO
Alicaforsen analog control	GAC-GCA-TCG-CGC-CTA-CAT-CA	PO
Alicaforsen analog sense	TAG-CGG-ATG-CCA-GCT-TGG-GC	PO
Alicaforsen-1-mod	GCC-CAA-GCU*-GGC-AUC-CGT-CA	PS
Alicaforsen-1-mod control	GAC-GCA-U*CG-CGC-CUA-CAU-CG	PS
Alicaforsen-2-mod	GCC-CAA-GCU*-GGC-AUC-CGU*-CA	PS
Alicaforsen-2-mod control	GAC-GCA-U*CG-CGC-CUA-CAU*-CG	PS
Alicaforsen-3-mod	GCC-CAA-GCU*-GGC-AU*C-CGU*-CA	PS
Alicaforsen-3-mod control	GAC-GCA-U*CG-CGC-CU*A-CAU*-CG	PS
SiRNA antisense	UAG-AGG-UAC-GUG-CUG-AGG-C-dTdT	
SiRNA antisense control	GGU-CAG-ACG-AGU-GAG-UUC-G-dTdT	
siRNA sense	GCC-UCA-GCA-CGU-ACC-UCU-A-dTdT	
siRNA sense control	CGA-ACU-CAC-UCG-UCU-GAC-C-dTdT	
siRNA-1-mod antisense	UAG-AGG-UAC-GU*G-CUG-AGG-CdTdT	
siRNA-1-mod sense	GCC-UCA-GCA-CGU*-ACC-UCU-AdTdT	
siRNA-2-mod antisense	UAG-AGG-U*AC-GUG-CU*G-AGG-CdTdT	
siRNA-2-mod sense	GCC-U*CA-GCA-CGU-ACC-U*CU-AdTdT	
siRNA-3-mod antisense	U*AG-AGG-U*AC-GUG-CU*G-AGG-CdTdT	
siRNA-3-mod sense	GCC-U*CA-GCA-CGU*-ACC-UCU*-AdTdT	

[Table 5] Sequences of DNA (alicaforsen, alicaforsen analog and alicaforsen with modifications), siRNA (siRNA and siRNA with modifications, each in sense and antisense form), their controls (equivalent to its oligonucleotide, contained several mismatches but the same number of bases and did not match any known human gene sequence) and sense forms of alicaforsen and alicaforsen analog. Alicaforsen analog has a similar nucleic acid structure as alicaforsen, but analog consists of a non-bridging oxygen atom in the phosphodiester backbone (PO). The non-bridging oxygen is replaced by sulphur atom in alicaforsen, therefore it is called phosphorothioate (PS).

The phosphoroamidite method is a solid support based techniques, which uses CPG (Controlled Pore Glass) as solid phase. The first base at the 3'end is bound to the CPG and the oligomer is built up by adding building blocks step by step via the phosphate backbone. With this method it is easily possible to synthesize also modified oligonucleotides such as phosphorothioate molecules (S-DNA). It has been shown to be the most efficient approach in modern oligonucleotide synthesis (26).

The phosphoroamidite synthesis [see Figure 12] consists of the following four steps: [A] deprotection, [B] coupling, [C] capping and [D] oxidation. Firstly, the 5'DMT-protection group is released by acid cleavage. After this detritylation [A] with trichlor-acetic acid in dichloromethane the synthesis continues with the most crucial reaction step coupling [B] of the next nucleoside phosphoramidite. This was carried out using the catalyzing solvents tetrazol or tetraethylthiuramidsulphide (TETD) for S-DNA, both in anhydrous acetonitrile. After coupling, unreacted 5'-hydroxy groups are capped by catalyzed acetylation [C]. Properly coupled groups (95%) are oxidized [D] by iodine. At the end the nucleotide is cleaved from solid support by concentrated ammonia solution and kept at 55°C for 8 hours for complete deprotection (26).

The 2'-O-lysyl-aminohexyl uridine modifications were synthesized prior to its incorporation in the alicaforsen oligonucleotide via the phosphoramidite method (26).
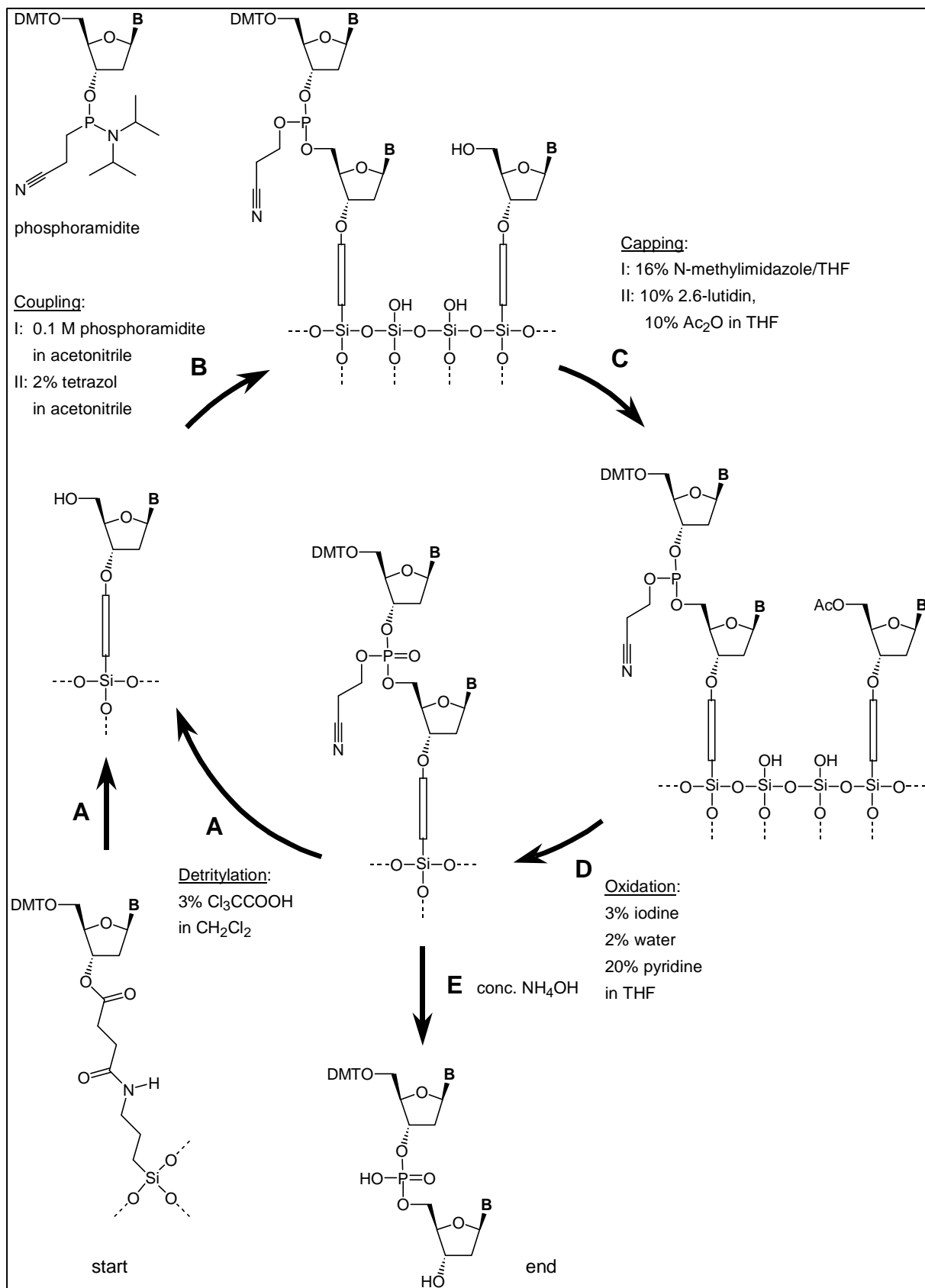
[Figure 12] The phosphoramidite method. Adapted from Urban E and Noe CR (29).

4.1.1. Synthesis and purification of DNA sequences

Bases (adenosine, guanine, thymine and cytosine) were dissolved in dried acetonitrile and put on the DNA synthesizer. Chemical synthesis was done in the 3' → 5' direction in contrast to biological systems (and hence the first base to be coupled is the last in the sequence).

At the end of the synthesis each solid product was left at $55°C$ overnight with ammonia for deprotection and purification. Nitrogen gas was bubbled through each solution to remove the ammonia. Then 1M triethylammoniumacetate (TEAA) buffer and acetic acid were added until the solution was pH neutral. Oligonucleotides were purified using solid phase extraction cartridges. The cartridge was first washed with acetonitrile and equilibrated with 1M TEAA. The oligonucleotide was applied three times to the cartridge and then the cartridge was washed with TEAA and water. 2% Trifluoro acetic acid was applied for detritylation and then the cartridge was washed again with water. The oligonucleotide was then eluted with 30% acetonitrile and dried using in a rotavapor.

For the ethanol precipitation the dried oligonucleotide was dissolved in a minimum amount of water and transferred to an Eppendorf tube. For crystallization 7.5N Ammonium acetate was added and the tube was filled with ethanol. After a short shaking, the tube was kept at 20°C overnight. The precipitate was centrifuged for 10 min at 15000rpm and 4°C. The supernatant was removed and the remaining precipitate was dissolved in 20 µl of water. Concentrations of the samples were determined using a spectrophotometer by measuring OD_{260}. This was done before and after purification with the Sep-Pak cartridge by diluting 50 µl in 1000 µl. Also after centrifugation, 1 µl of the oligonucleotide solution was diluted in 1000 µl water for determination of concentration. The molar extinction coefficients were calculated as sum of the bases contained in the respective sequences: $\varepsilon = (Ax15400) + (Gx11700) + (Cx7300) + (Tx8800)$. The final concentration of oligonucleotide was calculated and diluted to a 1mM stock solution, which was used for cell culture experiments.

4.1.2. Synthesis of siRNA sequences

The bases required adenosine, guanine, uridine and cytosine were dissolved in dried acetonitrile and applied to DNA synthesizer. The dU solution was placed in the dT position for ease of synthesis while the dT solution was placed in the X position. All chemicals were

prepared with RNAse free water ammonia-ethanol solution. Ammonia-ethanol solution was added to each of the solid phase bound RNA products and the solutions were left at $55°C$ for two hours for de-protection. Samples were washed with a solution of water:acetonitrile:ethanol (1:1:3) and subsequently the solvent was removed using a rotavapor. The residue was dissolved in tetrabutyl ammonium fluoride (1M in THF), and left overnight for cleavage of 2'protecting group. The next day TEAA was added to each flask to stop the raction and the liquid was reduced to half of the volume by a rotavapor. A Sep-Pak cartridge was used as described above to desalt the samples.

Each RNA precipitate was centrifuged and concentrations were determined by UV spectrophotometery. Again 1mM stock solutions were prepared for cell culture tests.

4.1.3. Synthesis of modifications

The synthesis of modificated oligonucleotides was performed in the same way. However, prior to the synthesis the modified uridine has been synthesized applying the 2'-O-aminohexyl linker method. A protected lysine building block was tethered to 2'-O-aminohexyluridine to produce a molecule that was then converted to the phosphoramidite.

4.2. Proof of purity

Electrophoresis is used to perform proof of purity. ODNs are examined by acrylamide gel electrophoresis for their purity.

Gelelectrophoresis

Initially the gel was prepared (see Materials). To dissolve urea it was necessary to heat in the microwave oven for 2 or 3 seconds at 400W. Then the gel was left to cool for 3 minutes. The finished gel was poured to the edge of the stand. Finally a comb for 10 pockets was inserted in the gel and was left to polymerise for 50 minutes.

Subsequently the electrophoresis was pre-run without any ODNs at 200V for 30 minutes. Solution of tris borate EDTA (TBE) was used as a buffer for both tanks of the electrophoresis instrument. Before starting the electrophoresis gel pockets were washed with TBE buffer in order to remove any unpolymerised material.

After the electrophoresis without ODNs 1.5 µl of samples were mixed with 10 µl of formamide buffer. Diluted proteins were heated at 95°C for 3 minutes and immediately put on ice. The gel pockets were washed with TBE buffer again to remove unpolymerised material. Then the samples were injected in to the pockets with a micro-syringe. 5 µl of marker (xylene cyanol and bromophenolblue) was applied to one pocket. The electrophoresis was run at 200V for 1.5 hour. After that time the bond of brompohenolblue (shows about 10 bases) reached 2/3 of the gel. The bond of xylene cyanol (shows 28 bases) stayed on the 1/3 of the gel.

For control the gel was put on TLC silicagel plate. Bonds of ODNs were seen under the UV-lamp at 254 nm.

Methylene blue-colouring

A fixing solution was used to attach ODNs on the gel. The whole process proceeded slowly on a shaker and lasted 15 minutes. Afterwards the gel was washed with water.

Then ODNs were stained in 0.1% methylene blue solution (in sodium acetate solution). It took 30 minutes till bonds were recognised and the colouring was stopped.

The gel was washed in distilled water five times for 10minutes to eliminate the colour from the background of the gel.

4.3. Cell culture

ECV 304 cells were maintained in PBMEC medium without fibronectin (PBMEC-FIB). Culture medium was changed every second or third day. ECV304 cells were grown in gelatin-coated small sized (25 cm²) flasks and splitted in a ratio according to their confluence. Splitting was done by trypsin. After washing and trypsinization the fresh medium was added according to the splitting ratio of cells. The cell suspension was resuspended and passaged to another cell culture flask. Because of culturing cells in the incubator at 37°C, all media had to be pre-warmed to that temperature before use. ECV304 were used between passages 150 and 200 and were monitored daily by a microscope.

One day (24 hours) before transfection, cells were passaged from middle sized (75 cm²) flasks to a 6-well gelatine-coated plate. Seeding was then proceeded like the splitting of cells. Cells were counted in a THOMA cell and 600000cells were then applied to each well on the 6-well plate.

4.4. Transfection

Transfection is the introduction of foreign DNA into cells. Transfection typically involves opening transient "holes" or gates in cells to allow the entry of extracellular molecules, like DNA or siRNA. A very efficient method of introducing foreign DNA into cell is the inclusion of the DNA into liposomes or cationic lipids, i.e. small, membrane-bounded bodies that are in some ways similar to the structure of a cell and can fuse with the cell membrane, releasing the DNA into the cell.

At the beginning of a transfection experiment a stock solution was prepared in an Eppendorf tube. ODNs of 1 mM were diluted 1:100 (1 μl of the ODN was mixed with 99 μl of PBS); to prepare an experiment stock solution with a concentration of 10 μM/100 μl.

End concentrations of ODNs used for experiments were 100 and 200 nM, for siRNA 25, 50, 75 and 100 nM, respectively. The experiment stock solution was diluted 1:10 with Optimem® (serum free medium, OM) to get 100 μl of new solution. After 30 minutes, Lipofectamin 2000 (LIPO) diluted in OM was prepared according to the manufacturer (for RNA: 5 μl LIPO/250 μl OM and for DNA: 10 μl LIPO/250 μl OM). 250 μl of LIPO solution was added to ODNs. The solution was left at room temperature to allow the formation of oligonucleotide lipid complex. After this incubation, 650 μl of pre-warmed OM, was carefully dropped to every eppendorf. See calculation below.

The 6-well plate was washed with 1ml of phosphate-buffered saline (PBS). Aliquots of 1 ml were carefully added to cells. After 4 hours of incubation at 37°C, 0.5 ml of medium without antibiotics (IMDM+F12 1:1/22, 5% NBS) was applied to every well. The 6-well plate was incubated overnight at the same temperature. The final amount of 1.5 ml per well is the optimal volume as shown by other experiments.

Contemplation:

concentration of the stock solution for 1 mM ODN every time:

  10 μM/100 μl (dilution 1:100; 1 μl of ODN + 99 μl of PBS)

concentration of the end solution of 1000μl from the stock solution:

  1 μM/100 μl (dilution 10:100; 10 μl of ODN + 90 μl of OM)

  0,1 μM/1000 μl = 100 nM/1000 μl (+ 250 μl of LIPO + 650 μl of OM)

Calculation by formula $c_1 \cdot V_1 = c_2 \cdot V_2$

concentration of the stock solution (c_2) for 1 mM ODN (c_1) 1:100 every time (dilution 1:100):

$$1 \cdot 10^{-3} \cdot 1 \cdot 10^{-6} = c_2 \cdot 100 \cdot 10^{-6}$$
$$10^{-9} = c_2 \cdot 10^{-4}$$
$$c_2 = 10^{-5}$$
$$c_2 = 10 \text{ μM (in 100 μl)}$$

concentration of the end solution (c_2) of 1000μl (V_2) from the stock solution (10 μM/100 μl):

$$10 \cdot 10^{-5} \cdot 100 \cdot 10^{-6} = c_2 \cdot 100 \cdot 10^{-6}$$
$$10^{-8} = c_2 \cdot 10^{-4}$$
$$c_2 = 10^{-5}$$
$$c_2 = 100 \text{ nM (in 1000 μl)}$$

4.5. Lysis

Lysis is the cell disruption that is used to release cell constituens such as DNA or RNA from the cell. In this experiments chemical lysis was done by lysis buffer together with protease inhibitor cocktail (PIC) (that inhibits acting of proteases, and therefore protein degradation) and dithithreitol (DTT) (cleaves disulfide bonds).

Firstly 2.5 mg of DTT and 5 μl of PIC were freshly added to an aliquot of lysis buffer. The 6-well plate was put on ice and afterwards each well was washed with 1 ml unsteril PBS. In every well 70 μl of lysis buffer acted 3 minutes. The solutions were scraped into Eppendorf tubes, which were centrifuged at 15000 rpm and 4°C for 20 minutes in order to remove cell debris (pellets). Supernatants containing the soluble proteins were collected and used for protein concentration determination by the Bradford assay. Protein solutions were stored in the freezer at - 80°C.

4.6. Bradford assay

With the Bradford assay protein concentrations were determined. Proteins were measured by a spectrophotometer at 595 nm. Eppendorf tubes comprised 1 ml Bradford reagent and appropriate volume of distilled water. As a standard 0.1% Bovine serum albumin (BSA) was used. Standard dilutions prepared standard as follows: 0:100 (the blind sample for auto-zero); 5:95; 10:90 and 20:80. All of these dilutions were measured twice and used for building up a

calibration curve. Dilutions of proteins were done as follows: 0, 5:99, 5; 1:99 and 2:98. Always six probes were measured in an interval of 5 minutes. At the end one set of standards were measured again as control [see Table 6]. With the help of a calibration curve and a graph concentrations [see Figure 13] were determined.

Label	Vol. Bradford reagent [ml]	Vol. 0.1% BSA [µl]	Vol. Milli-Q Water [µl]	Vol Sample [µl]	Extinction
Standard-0-A	1	0	100		0.000
Standar-0-B	1	0	100		0.006
Standard-2,5-A	1	5	95		0.215
Standard-2,5-B	1	5	95		0.216
Standard-5-A	1	10	90		0.393
Standard-5-B	1	10	90		0.405
Standard-10-A	1	20	80		0.677
Standard-10-B	1	20	80		0.650
Control	1		99.5	0.5	0.112
	1		99	1	0.170
	1		98	2	0.378
Alicaforsen 100 nM	1		99.5	0.5	0.152
	1		99	1	0.266
	1		98	2	0.404

[Table 6] Protein concentration determination of alicaforsen 100 nM.
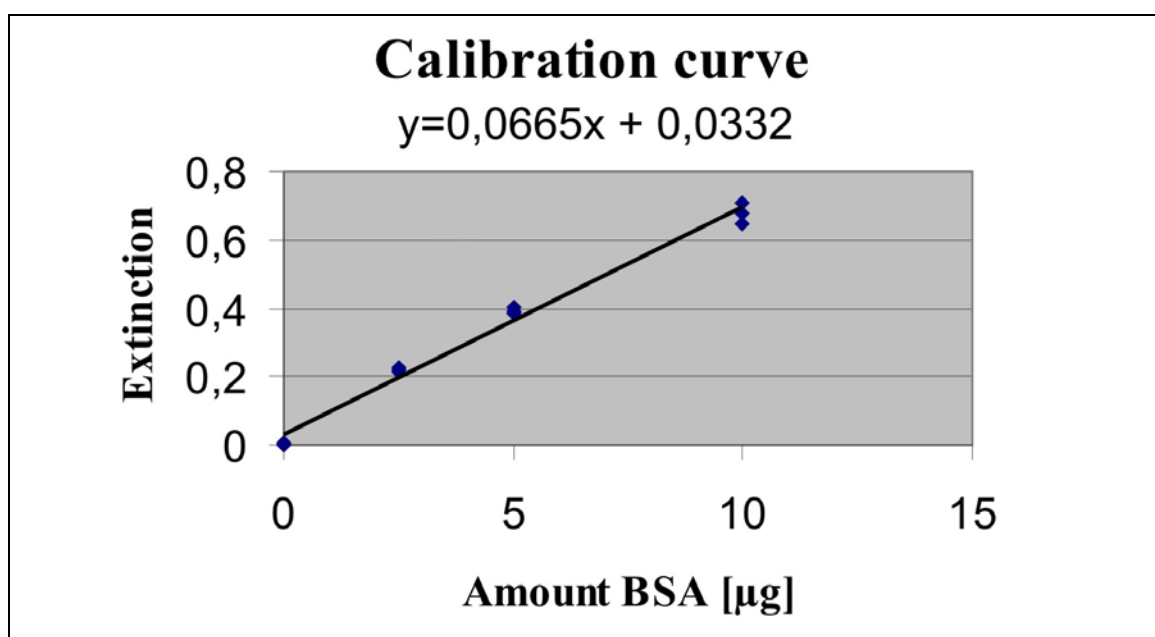
[Figure 13] Calibration curve of alicaforsen 100 nM.

4.7. Western Blot

The ECL plus western blot (Amersham Biosciences) is a non-radioactive method for the detection of immobilized specific antigens conjugated to Horseradish Peroxidase (HRP) labelled antibodies.

The western blot method consists of several steps: gel electrophoresis, blotting procedure, blocking and incubation with antibodies. Initially, during gel electrophoresis proteins are separated according to their molecular weight corresponding to different travelling speeds through the gel pores. Smaller proteins move faster than bigger proteins. During the blotting procedure proteins are transformed from the gel on a PVDF blotting membrane. The subsequent blocking solution avoids unspecific binding.

SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

Before electrophoresis was started, separating and stacking gels were prepared. Separating gel was on the bottom of the gel stand and was filled up until 1 cm from the edge of the stand. Into the empty space water saturated butanol was poured to make one horizontal line with the edge of the gel stand. It was left to polymerise for about 50 minutes. After this time butanol was washed away and the stacking gel was poured. Into stacking gel a comb consisting of 10 pockets (special compartments for samples) was inserted. This time the gel was polymerised for about 60 minutes.

During the polymerisation the samples were prepared. According to concentrations determined with the Bradford assay, 20 µg of protein were diluted up to 20 µl with Laemmli buffer containing 6.2 mg DTT. Samples were heated up to 95°C, at 350 rpm for 10 minutes in a thermocycler and afterwards they were immediately put on ice. If necessary, samples were spun down at 4°C and 11000 rpm.

The electrophoresis instrument was composed of two tanks. The first bigger tank was filled with anode buffer (diluted 1:10) and the second smaller tank with cathode buffer (buffer description see Materials) separated by the gel. A molecular weight standard (Precision plus Protein all blue standard, Bio-Rad) was used containing bonds at 10, 15, 20, 25, 37, 50, 75, 100, 150, 200 kDa. With a micro-syringe 2 µl of marker were applied in two gel pockets and the remaining pockets were filled with 20 µl of samples. Electrophoresis was run at 125 V for 80 to 85 minutes. After the electrophoresis the separated marker bonds were visible.

Blotting

Initially the blotting membrane was wetted slightly with methanol. Then the membrane, two filter pads and the gel were washed in blotting buffer (buffer description see Materials) for 5 minutes. In the blotting machine a sandwich was formed as follows: the filter pad, the membrane, the gel and the second filter pad. The blotting machine was carried out at 50 mA per gel for 60 minutes.

Blocking the membrane

After one hour the membrane was removed from the blotting machine and was put into the about 20 to 30 ml of blocking solution (solution's description see Materials) and shaken for 15 minutes. Afterwards the basin was covered with the alumina foil and stored in the refrigerator overnight.

Incubation with antibodies

The target protein ICAM-1 is specifically recognized by the primary antibody (Ab). The secondary Ab, which is connected to HRP binds to the primary Ab. For the final detection of proteins ECL plus solution is applied. During this process the substrate for HRP, an acridium ester, is produced. The acridium ester is cleaved. Chemiluminescence, which is produced by the cleavage, can be detected by autoradiography (films).

Incubation of primary Ab

The membrane was washed four times for 8 minutes each with 0.1% TBST. After the washing, primary Ab in buffer was prepared. The dilution factor was determined empirically for each Ab. Anti-actin Ab (from rabbit) was finally used in a concentration of 0.6 μg/ml (dilution 1:1000) and anti-ICAM-1 Ab (from mouse) in 1 μg/ml (dilution 1:700). The membrane was cut in two pieces at 50 kDa, because actin has 42 kDa and ICAM-1 has 85 kDa. Each part of the membrane was put into a basin with the corresponding primary Ab in buffer actin – anti-actin Ab and ICAM-1 – anti-ICAM-1 and placed on a shaker. Both basins were shaken slowly for 60 minutes.

Secondary AB incubation

After the incubation time the membrane was washed four times for 8 minutes with 0.1% TBST. After the washing, secondary Ab dilution was prepared: goat anti-rabbit IgG-HRP in

concentration of 0.1 µg/ml was diluted 1:10000 and linked to anti-actin; goat anti-mouse Ab was diluted 1:5000 and was bound to ICAM-1. Both pieces of the membrane with the corresponding secondary Ab (anti-actin Ab - goat anti-rabbit IgG-HRP and anti-ICAM-1 Ab – goat anti-mouse Ab) were shaken gently for 60 minutes.

Detection

Last washing was done four times for 8 minutes with 0.1% TBST. After the washing, developing solution was prepared. The detection solution of ECL plus was mixed in a ratio 40:1 (3 ml A solution + 75 µl B solution). The membrane was incubated for 5 minutes, put into a film cassette – the two membrane pieces again put together. It was developed on, very sensitive (Amersham Hyperfilm ECL) and a less sensitive film (Kodak).

5. RESULTS

Proof of purity

All samples were tested to determine their purity; proof of purity was used for this evaluation. By all samples only one clear bond was seen under the UV lamp [see Figure 14], therefore all sequences were pure.
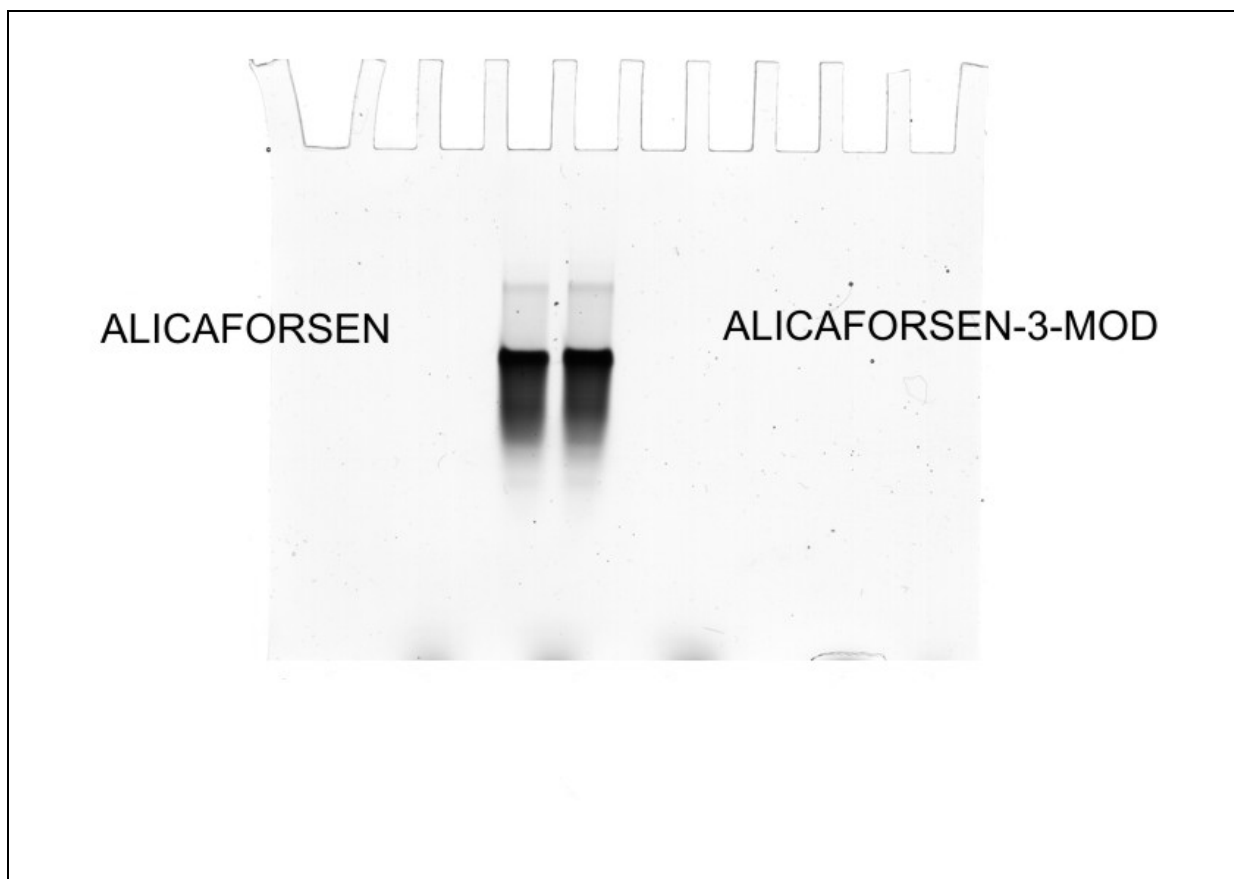
[Figure 14] Proof of purity: alicaforsen and alicaforsen with 3U*.

Best concentration of ICAM-1 Ab

For the primary Ab the optimal concentration had to be tested. Therefore concentrations of 1:200, 1:500 and 1:700 were examined [see Figure 15]. Bonds were seen in an optimal relation when diluted 1:700.
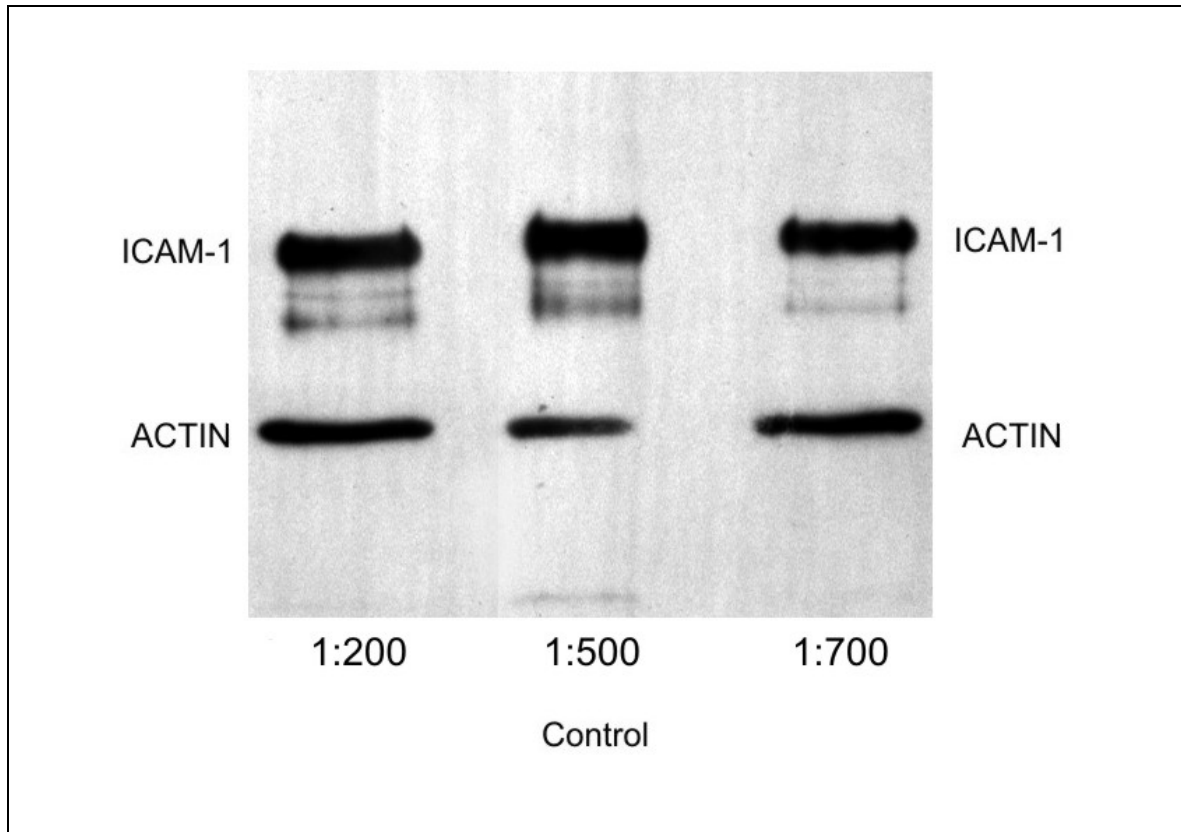

[Figure 15] Determination of the best concentration of ICAM-1 Ab.

Calculations of ICAM-1 inhibition

Inhibitions of ICAM-1 were calculated and related to actin and to untreated cells. Actin is a protein, which is equally expressed in every cell and is uniform.

For each oligonucleotide, a control was synthesised and tested that included several mismatches, but the same number of bases, and did not match any known human gene sequence. Controls, containing the same amount but different arrangement of bases should not show any inhibiting effect and should therefore verify the specific effect on alicaforsen sequences proving that shown inhibitions [see Figure 16] were not accomplished by some other factor. All these control sequences were tested only once.
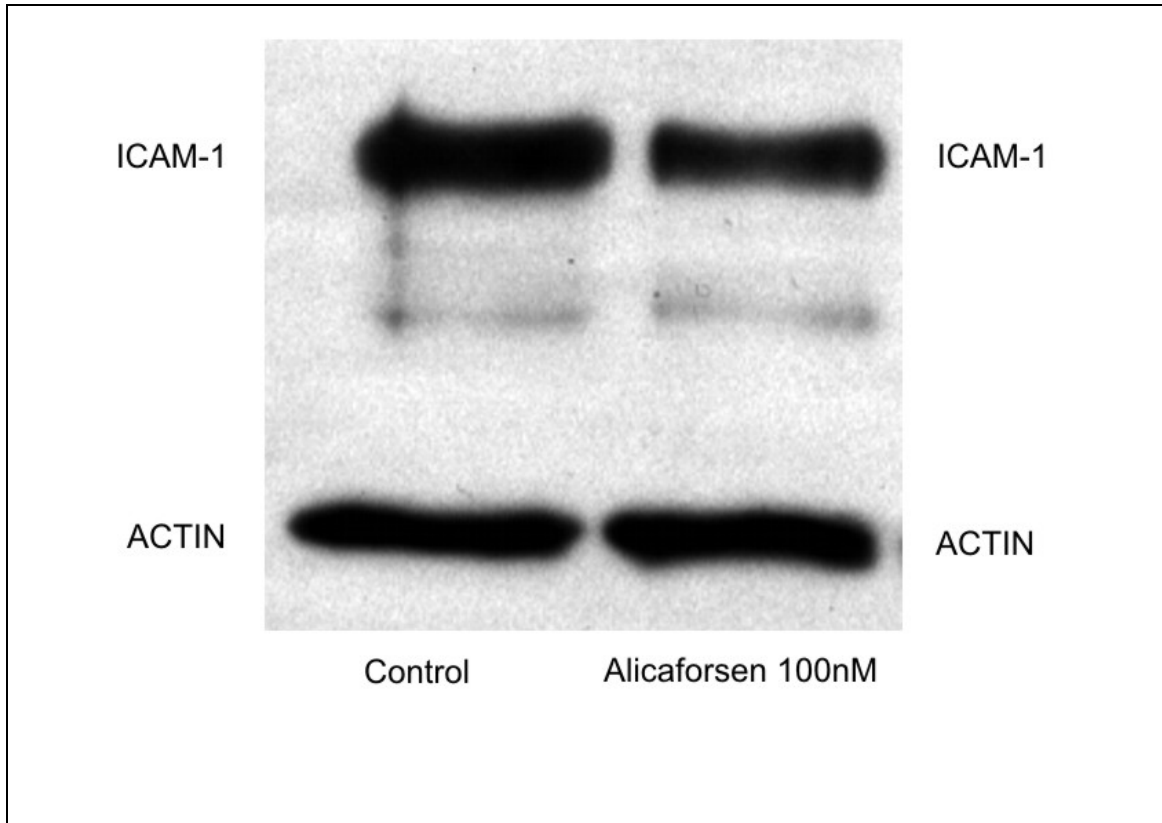
[Figure 16] Alicaforsen 100 nM (40.79% of inhibition).

DNA substances were tested in 100 and 200 nM. These concentrations are commonly used for gene silencing experiments. Three independent replicates were performed with each type of molecule in order to achieve statistical relevance. If the number of inhibitions did not fit the pattern, it was marked as an outlier and was excluded from the calculation. Inhibitions of ICAM-1 were quantified using the software program Quantity One (Bio-rad) [see Table 7] and followed by calculations [see Table 8].

Gel name : WB 6 90sec 24.1.07			
Name	Volume	Adj. Vol.	**Adj. Vol.[%]**
ICAM-1			
Control	39.30	25.01	10.40
Alicaforsen 100 nM	26.82	15.75	6.55
ACTIN			
Control	33.44	21.49	8.94
Alicaforsen 100 nM	34.89	22.85	9.50

[Table 7] Values detected by program Quantity One.

Gel name : WB 6 90sec 24.1.07				
Name				
ICAM-1	ICAM-1[%]	ICAM-1 related to actin [%]	**ICAM-1 inhibition [%]**	
Control	100.00	100.00	0.00	**Control**
Alicaforsen 100 nM	62.95	5.21	40.79	**Alicaforsen 100 nM**
ACTIN	ACTIN%			
Control	100.00			
Alicaforsen 100 nM	106.33			

[Table 8] Values quantified from values in Table 7, calculation see below.

Calculation:

ICAM-1[%]:

Adj.vol. of ODN targeted to ICAM-1 [%]/Adj.vol. of control [%] targeted to ICAM-1

ACTIN [%]:

Adj.vol. of ODN targeted to actin [%]/Adj.vol. of control [%] targeted to actin

ICAM-1 related to actin [%]: ICAM-1[%]/actin [%]

**ICAM-1 inhibition [%]:** 100- ICAM-1 related to actin [%]

Allocated controls to each Oligonucleotide

Controls (alicaforsen control, alicaforsen analog control, alicaforsen-1-mod control, alicaforsen-2-mod control, alicaforsen-3-mod control, siRNA antisense control and siRNA sense control) showed no inhibition.

Alicaforsen and alicaforsen analog

IDENTITY	INHIBITION %	MEAN VALUE %	STANDARD DEVIATION %
Alicaforsen 100 nM	40.8	46.6	7.3
	54.8		
	44.3		
	60.2 (outlier)		
Alicaforsen 200 nM	52.4	45.4	6.8
	38.9		
	44.8		
	30.9		
Alicaforsen analog 100 nM	26.5	25.2	1.7
	25.8		
	23.3		
	33.7 (outlier)		
Alicaforsen analog 200 nM	16.6	14.9	3.4
	17.1		
	11.0		

[Table 9] Gene expression of ICAM-1 by alicaforsen and alicaforsen analog in 100 and 200 nM.
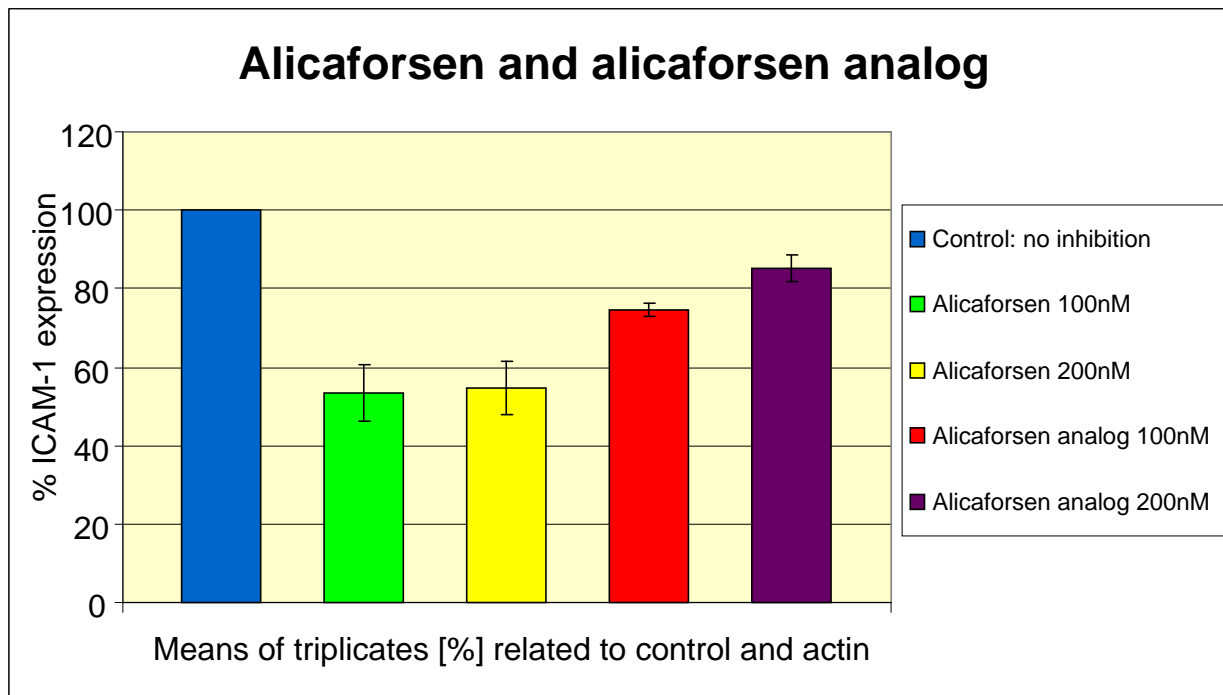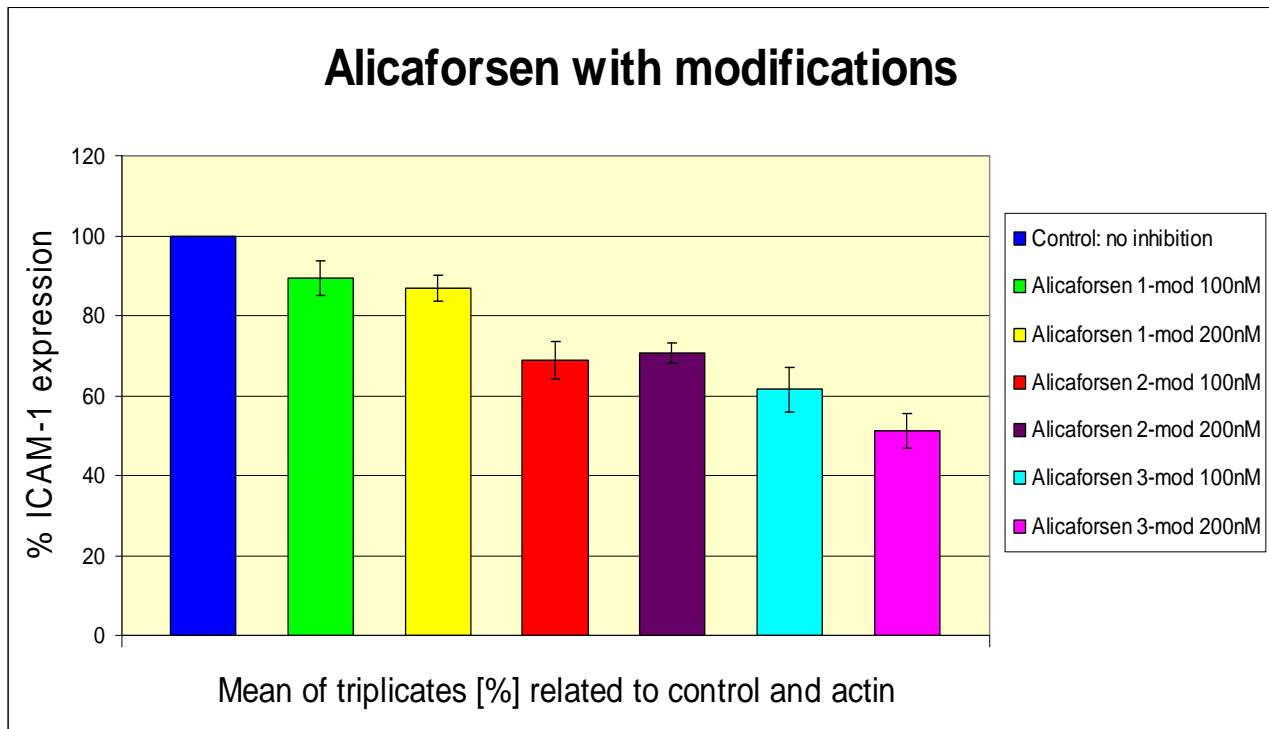

[Figure 17] Gene expression of ICAM-1 by alicaforsen and alicaforsen analog in 100 and 200 nM.

Alicaforsen with modifications

IDENTITY	INHIBITION %	MEAN VALUE	STANDARD DEVIATION
Alicaforsen-1-mod 100 nM	15.6	10.7	4.3
	9.1		
	7.6		
Alicaforsen-1-mod 200 nM	14.1	13.2	3.2
	9.6		
	15.8		
Alicaforsen-2-mod 100 nM	34.5	31.1	4.7
	27.8		
	43.4 (outlier)		
Alicaforsen-2-mod 200 nM	30.2	30.0	2.4
	29.9		
	26.0		
	31.7		
Alicaforsen-3-mod 100 nM	41.0	38.5	5.7
	30.4		
	39.3		
	43.3		
Alicaforsen-3-mod 200 nM	49.4	48.9	4.4
	53.6		
	43.0		
	49.5		

[Table 10] Gene expression of ICAM-1 by alicaforsen 1U*, 2U* and 3U* in 100 and 200 nM.
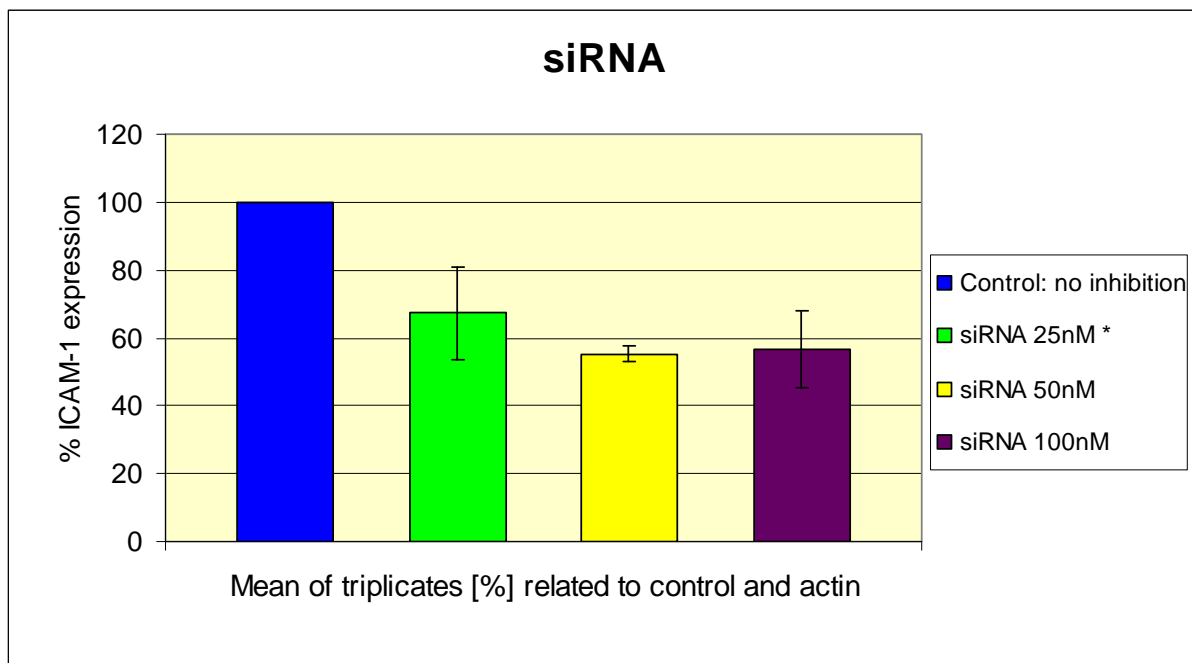

[Figure 18] Gene expression of ICAM-1 by alicaforsen 1U*, 2U* and 3U* in 100 and 200 nM.

siRNA

IDENTITY	INHIBITION %	MEAN VALUE	STANDARD DEVIATION
siRNA 25 nM	42.4	32.7	13.8
	22.9		
siRNA 50 nM	43.0	44.7	2.2
	43.9		
	47.2		
siRNA 100 nM	34.5	43.4	11.1
	39.7		
	55.9		
	59.9 (outlier)		

[Table11] Gene expression of ICAM-1 by siRNA in 25, 50 and 100 nM.

* Mean was calculated from duplicates

[Figure 19] Gene expression of ICAM-1 by siRNA in 25, 50 and 100 nM.

siRNA with one modification

IDENTITY	INHIBITION %	MEAN VALUE	STANDARD DEVIATION
siRNA-1-mod 25 nM	21.7	27.2	4.9
	31.3		
	28.5		
siRNA-1-mod 50 nM	33.6	32.9	0.9
	33.2		
	31.8		
siRNA- 1-mod 75 nM	13.5	19.7	7.7
	17.2		
	28.3		
siRNA -1-mod 100 nM	25.1	23.6	2.1
	22.2		

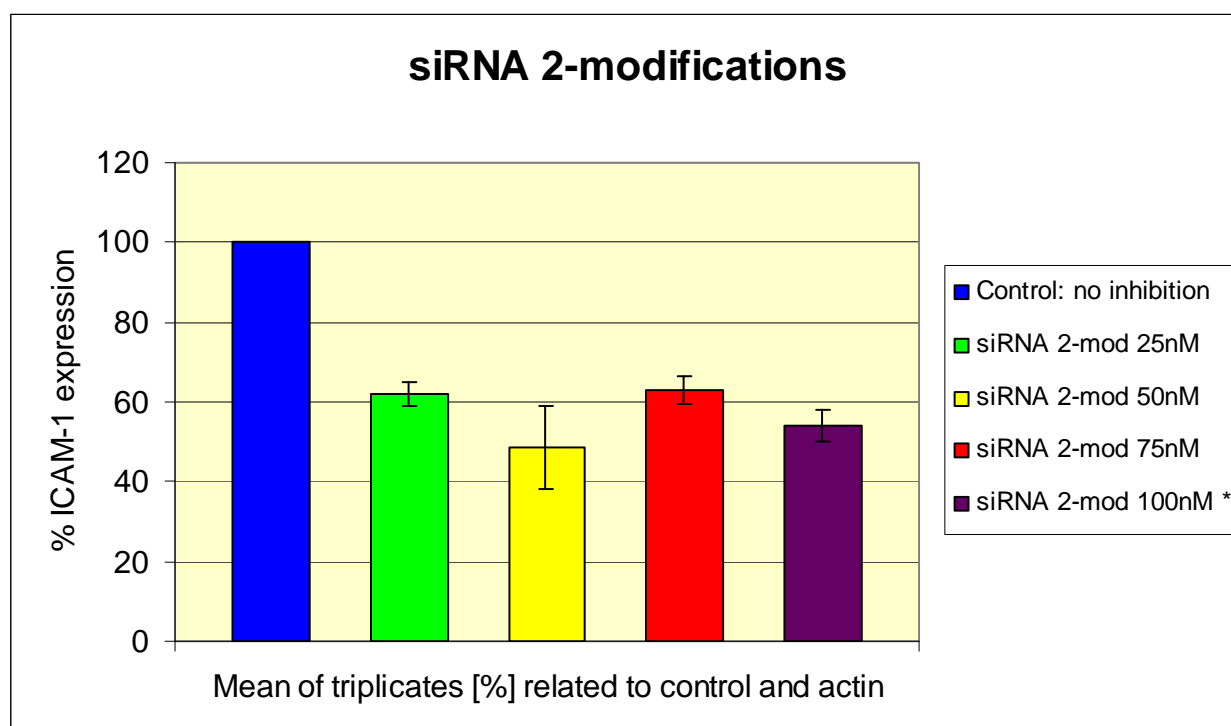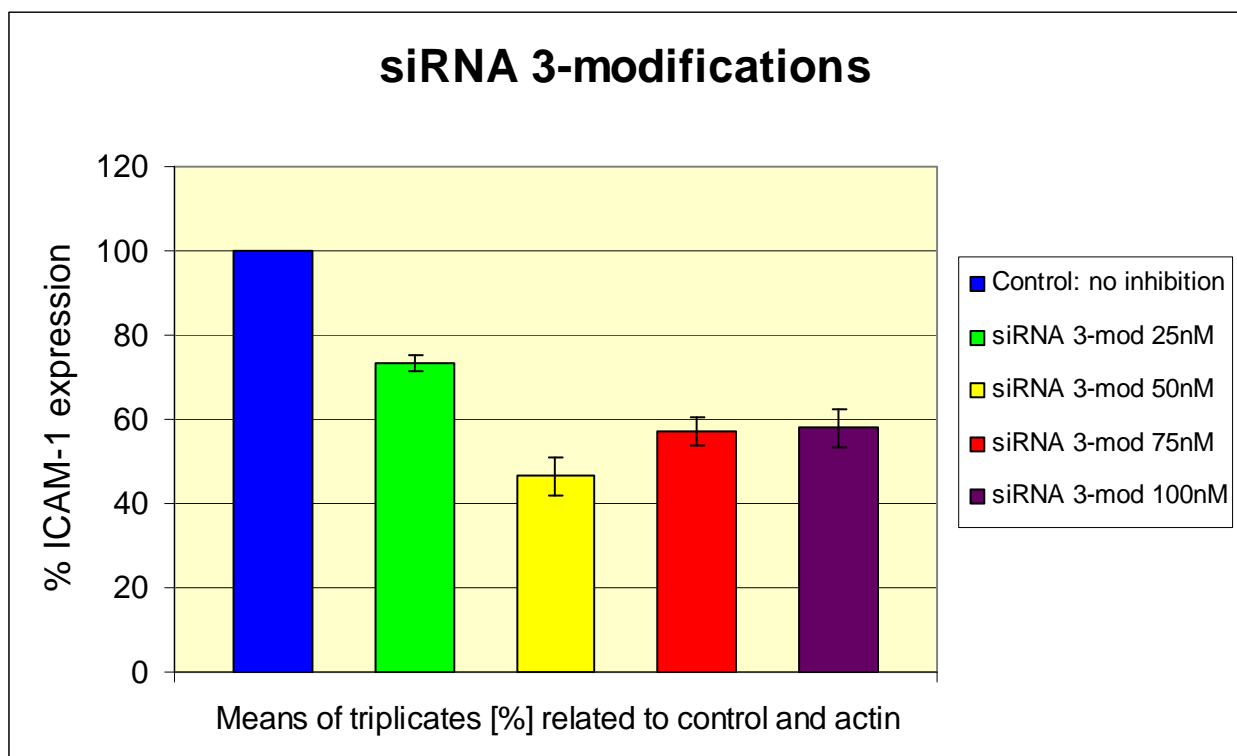[Table 12] Gene expression of ICAM-1 by siRNA 1U* in 25, 50, 75 and 100 nM.
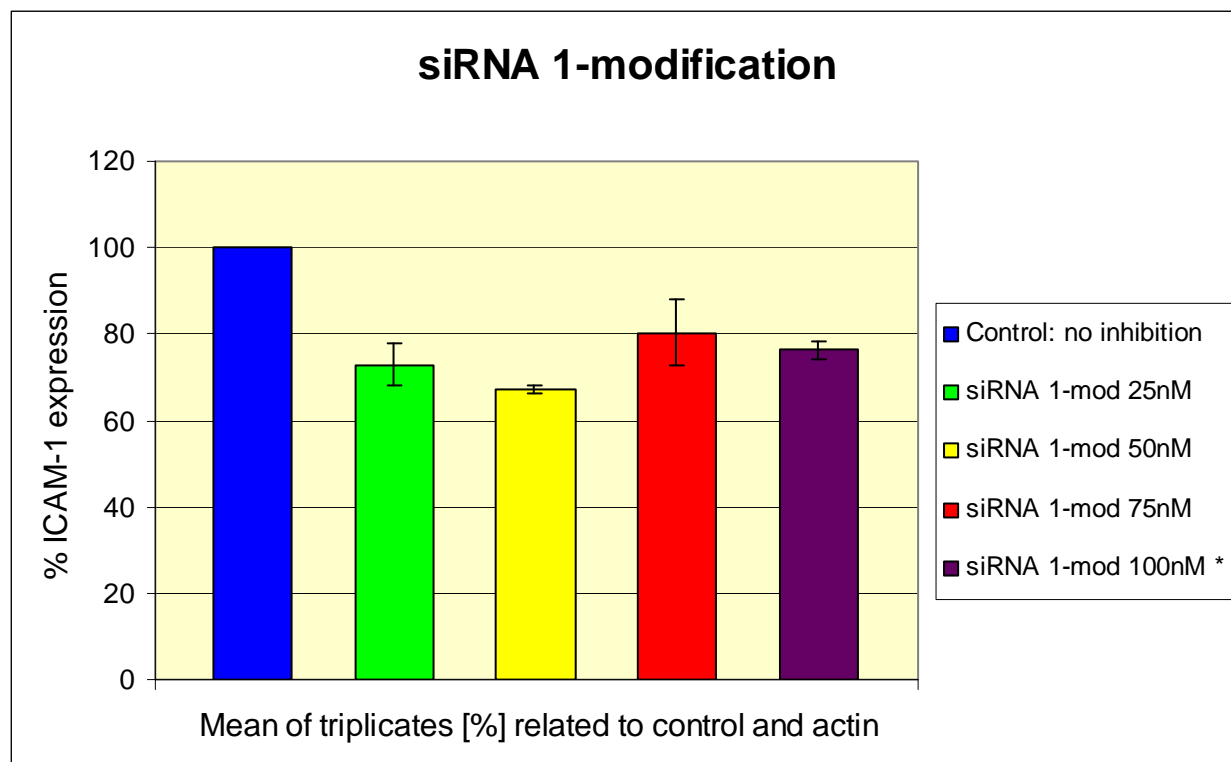
* Mean was calculated from duplicates

[Figure 20] Gene expression of ICAM-1 by siRNA 1U* in 25, 50, 75 and 100 nM.

siRNA with 2 modifications

IDENTITY	INHIBITION %	MEAN VALUE	STANDARD DEVIATION
siRNA-2-mod 25 nM	39,6	38.1	3.1
	34,6		
	40,2		
	31,2 (outlier)		
siRNA-2-mod 50 nM	54,7	51.5	10.6
	57,4		
	39,3		
siRNA- 2-mod 75 nM	35,0	37.0	3.6
	41,2		
	34,8		
siRNA -2-mod 100 nM	43,0	45.8	3.9
	48,6		

[Table 13] Gene expression of ICAM-1 by siRNA 2U* in 25, 50, 75 and 100 nM.

[Figure 21] Gene expression of ICAM-1 by siRNA 2U* in 25, 50, 75 and 100 nM.

* Mean was calculated from duplicates

siRNA with three modifications

IDENTITY	INHIBITION %	MEAN VALUE	STANDARD DEVIATION
siRNA-3-mod 25 nM	24.5	26.6	1.9
	27.5		
	27.9		
siRNA-3-mod 50 nM	50.3	53.5	4.5
	56.6		
siRNA- 3-mod 75 nM	46.0	42.8	3.4
	39.3		
	43.0		
	47.1 (outlier)		
siRNA -3-mod 100 nM	39.9	42.0	4.5
	39.0		
	47.2		

[Table 14] Gene expression of ICAM-1 by siRNA 3U* in 25, 50, 75 and 100 nM.

[Figure 22] Gene expression of ICAM-1 by siRNA 3U* in 25, 50 75 and 100 nM.

Graphs demonstrate these results:

alicaforsen shows better efficiency than alicaforsen analog

alicaforsen 3U* shows better efficiency than alicaforsen 2U* and alicaforsen 1U*; alicaforsen 2U* shows better efficiency than alicaforsen 1U*

alicaforsen 3U* shows similar efficiency than alicaforsen; alicaforsen 1U* and 2U* show less efficiency than alicaforsen

siRNA shows similar efficiency like alicaforsen

siRNA 2U* shows better efficiency than siRNA 3U* and siRNA 1U*; siRNA 3U* shows better efficiency than siRNA 3U*

siRNA 2U* and 3U* show better efficiency than siRNA; siRNA 1U* shows less efficiency than siRNA

siRNA 2U* and 3U* show better efficiency than alicaforsen 1U*, 2U* and 3U*, siRNA 1U* shows better efficiency than alicaforsen 1U* and 2U*

siRNA 2U* and 3U* show better efficiency than alicaforsen; siRNA 1U* shows less efficiency than alicaforsen

6. DISCUSSION

For this work, ICAM-1 was selected as target molecule and the cell line ECV 304 was used as an *in vitro* BBB. Both ODN and siRNA sequences were deducted from literature and had been used in other *in vitro* studies on different cell lines. The antisense oligonucleotide alicaforsen looks back to a decade of clinical development. It was verified that both ODN and siRNA were able to significantly reduce the levels of the target protein ICAM-1. The extent of down-regulation was in the range of 50% for both siRNA and alicaforsen. Concentrations of siRNA to achieve the same effect were lower than for ODNs.

DNA

It is known that only 1 out of 18-20 tested ODNs shows significant inhibition of target gene expression. Experimental approaches to find an effective ODN are extremely expensive, therefore semi-empirical rules were made on computer to design effective ODNs and identify target sequences. In one study (93) the software Oligo v.3.4 was used to predict structures of ODNs that suppressed ICAM-1. By applying these described structures, 17 of 34 ODNs showed significant inhibition (>50%) of ICAM-1 in endothelial ECV 304 cells cultured in medium 199. Alicaforsen was efficient showing 26-50% inhibition (93). These results were confirmed by present study: alicaforsen 100 nM suppressed ICAM-1 by 46.6%, alicaforsen 200 nM by 45.4%. Alicaforsen 100 nM and 200 nM (46.6 and 45.4%) were more efficient in gene silencing of ICAM-1 than alicaforsen analog in the same concentrations (25.2% and 14.9%). Alicaforsen analog has a similar nucleic acid structure like alicaforsen; analog contains a non-bridging oxygen atom in the phosphodiester backbone. The non-bridging oxygen is replaced by sulphur atom in alicaforsen, therefore it is called phosphorothioate. This exchange should cause better stability against enzymatic degradation.

As previous results (44, 94, 95, 96 and 97) have shown the 2'-lysine modification can be advantageous in cell uptake, nevertheless it remained uncertain if a 2'-lysine modification shows any increase in affinity for the ICAM-1 gene. Therefore it is the first time alicaforsen has been modified in the way of the 2'-lysine linker. Alicaforsen was modified by replacing thymine bases with 2'-lysine modified uridine (2'-O-aminohexyluridine) on one, two or three places in the molecule. As can be seen from the comparison of these data of modified oligonucleotides with inhibitions of alicaforsen and alicaforsen analog [see Table 9 and 10; see Figure 17 and 18], it can be clearly interpreted that the inhibition of alicaforsen 100 nM should be less than 46.6%. Dose dependence is determined by applying several decreasing

concentrations, which should show lower inhibitions according to the decreasing concentrations. Thus the inhibition of alicaforsen 100 nM is not used as it exceeds the concentration of alicaforsen 200 nM compared to other sequences used in this study. Alicaforsen analog 100 nM and alicaforsen 200 nM were compared with alicaforsen containing modifications [see Figure 23, 24 and 25]. Comparing modified and unmodified alicaforsen in a concentration of 100 nM [see Figure 23] it could be shown that the most efficient substance was alicaforsen 3U* (38.5%) followed by alicaforsen 2U* (31.1%), alicaforsen analog (25.2%) and alicaforsen 1U* (10.7%). The concentration of 200 nM was used with the same sequences [see Figure 24] and it showed similar dose dependence: alicaforsen 3U* (48.9%) had the best efficiency, then alicaforsen (45.4%), alicaforsen 2U* (30.0%) and alicaforsen 1U* (13.2%). From figures 17 and 18 it seems that the concentration of 200 nM was the better choice for gene silencing of ICAM-1. This result can be interpreted more clearly from the summary figure 25. There can be also demonstrated that the most efficient sequence in 200 nM was alicaforsen 3U*.
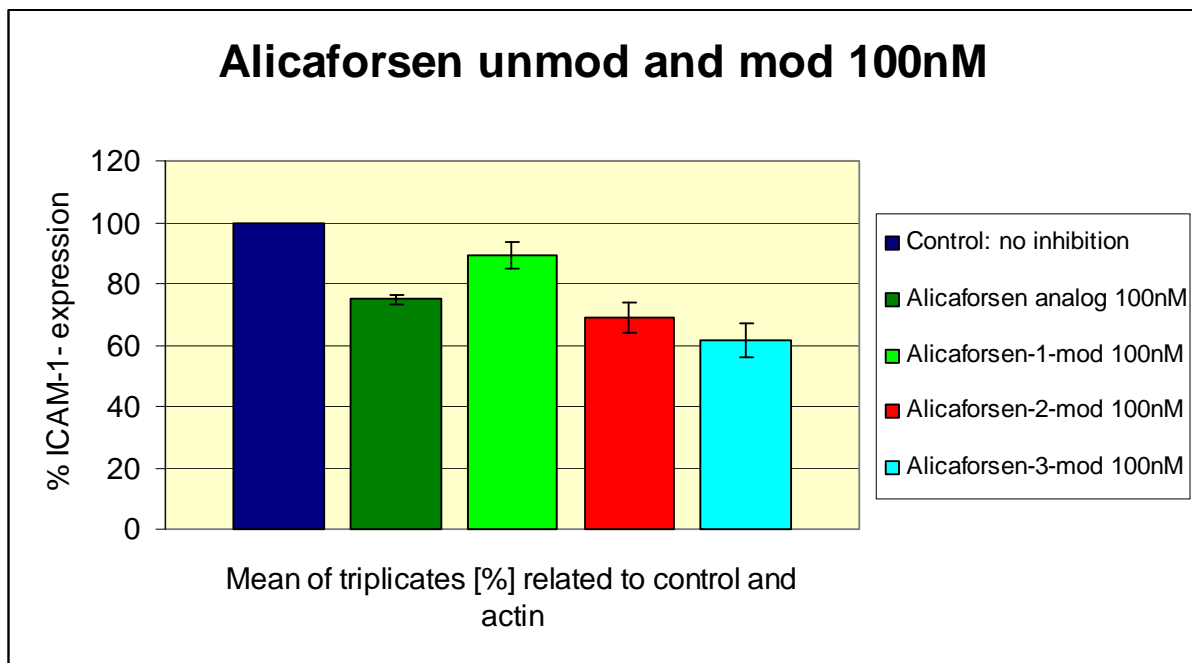
[Figure 23] Concentration 100 nM of alicaforsen analog, alicaforsen 1U*, alicaforsen 2U* and alicaforsen 3U*.
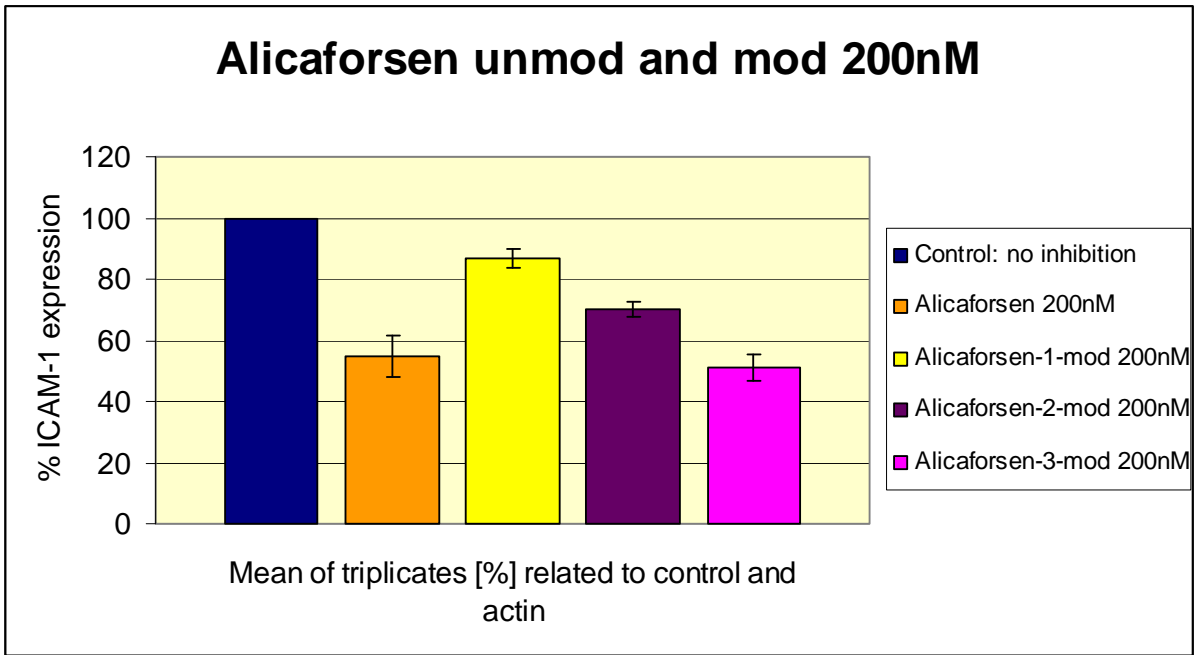
[Figure 24] Concentration 200 nM of alicaforsen, alicaforsen 1U*, alicaforsen 2U* and alicaforsen 3U*.
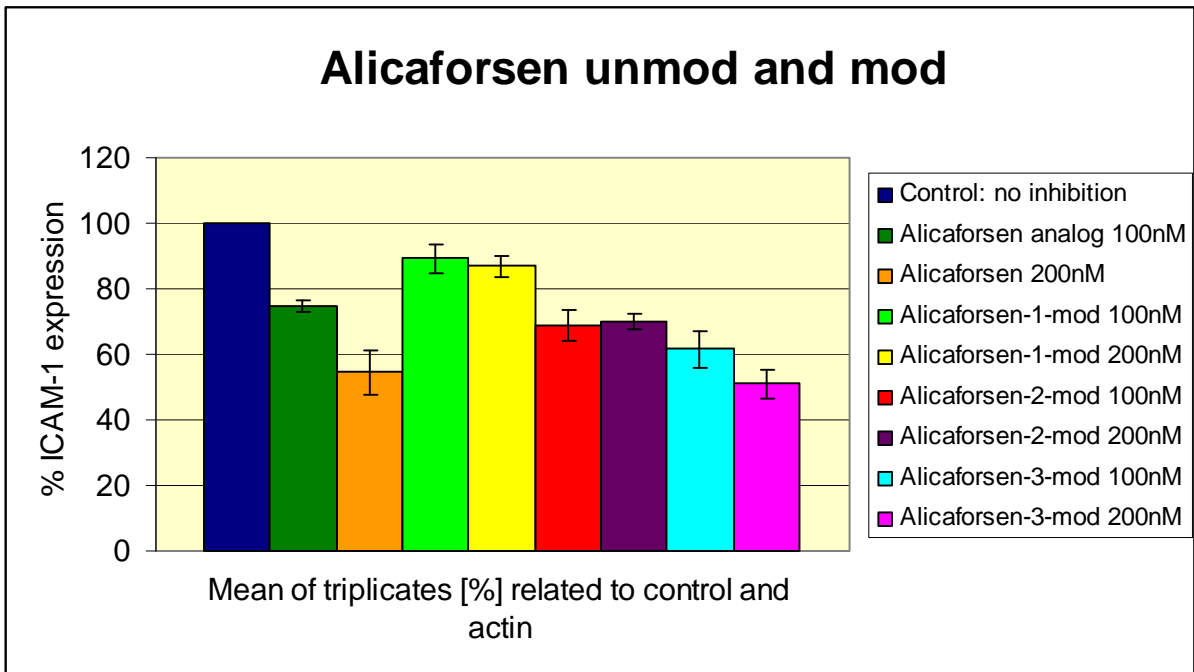
[Figure 25] Summary of concentrations: 100 and 200 nM (alicaforsen analog, alicaforsen, alicaforsen 1U*, alicaforsen 2U* and alicaforsen 3U*).
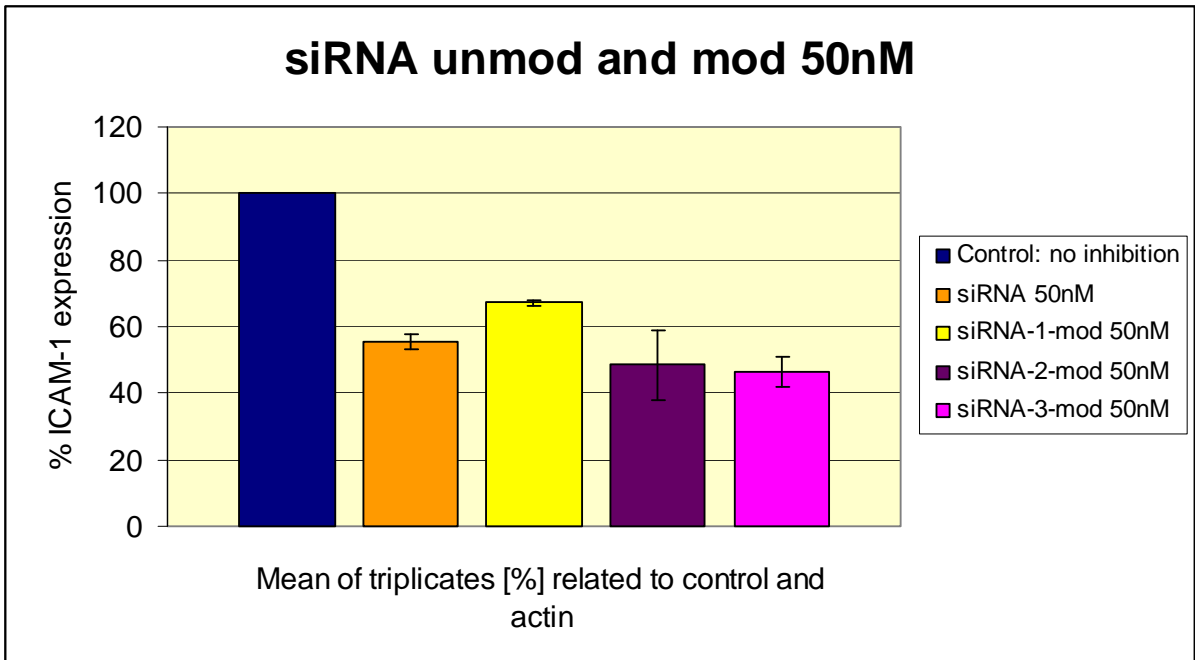
siRNA

siRNA is a quite new phenomenon and an increasing number of successful gene knockdown has been reported recently. In one study (98) siRNAs designed to target human E-selectin were transfected to HUVEC and attenuated E-selectin expression in HUVEC was activated by TNF-α, resulting in a reduced leukocyte adhesion. From this first observation in this field it can be suggested that this approach may provide a therapeutic option for inflammation and atherosclerotic disorders. Above mentioned project was performed on ECV 304 cells from a Japanese HUVEC culture. Cells had to be spontaneously immortalized and transformed (see 4.7.1.). Recently a first report on ICAM-1 expression by siRNA has been introduced. It stated ICAM-1 was effectively silenced by siRNAs on venous endothelial cells from human vena saphena magna specimens (99). In accordance with this publication siRNA sense, antisense and scrambled sequences were synthesized in this project. A comparison of the alicaforsen sequence using DNA and siRNAs against ICAM-1 is only described in T24 cells. According to the authors alicaforsen reduced ICAM-1 expression by 85%, siRNAs had no inhibitory effect at concentrations as high as 200 nM (100).

The modified siRNAs were more successful at inhibition than unmodified sequences. Dose dependence could be shown at concentrations of 25 and 50 nM [see Figure 26 and 27]. At 25 nM most effective sequence was siRNA 2U* (38.1%), subsequently siRNA (32.7%), siRNA 1U* (27.2%) and siRNA 3U* (26.6%). At 50 nM siRNA 3U* (53.5%) proved to have the best inhibition, followed by siRNA 2U* (51.5%), siRNA (44.7%) and siRNA 1U* (32.9%). From results in the summary figure 28 can be concluded that 50 nM was the best concentration for modified and unmodified siRNA and the most effective sequence in 50 nM was siRNA 3U*.
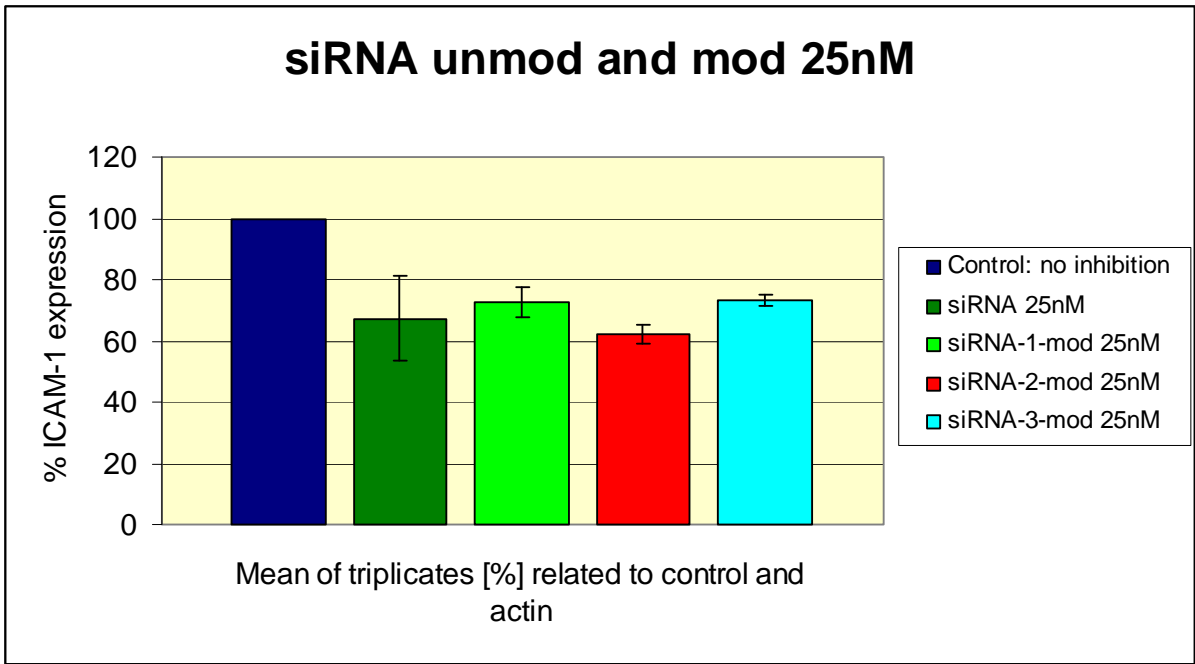
[Figure 26] Concentration 25 nM of siRNA, siRNA 1U*, siRNA 2U* and siRNA 3U*.

[Figure 27] Concentration 50 nM of siRNA, siRNA 1U*, siRNA 2U* and siRNA 3U*.
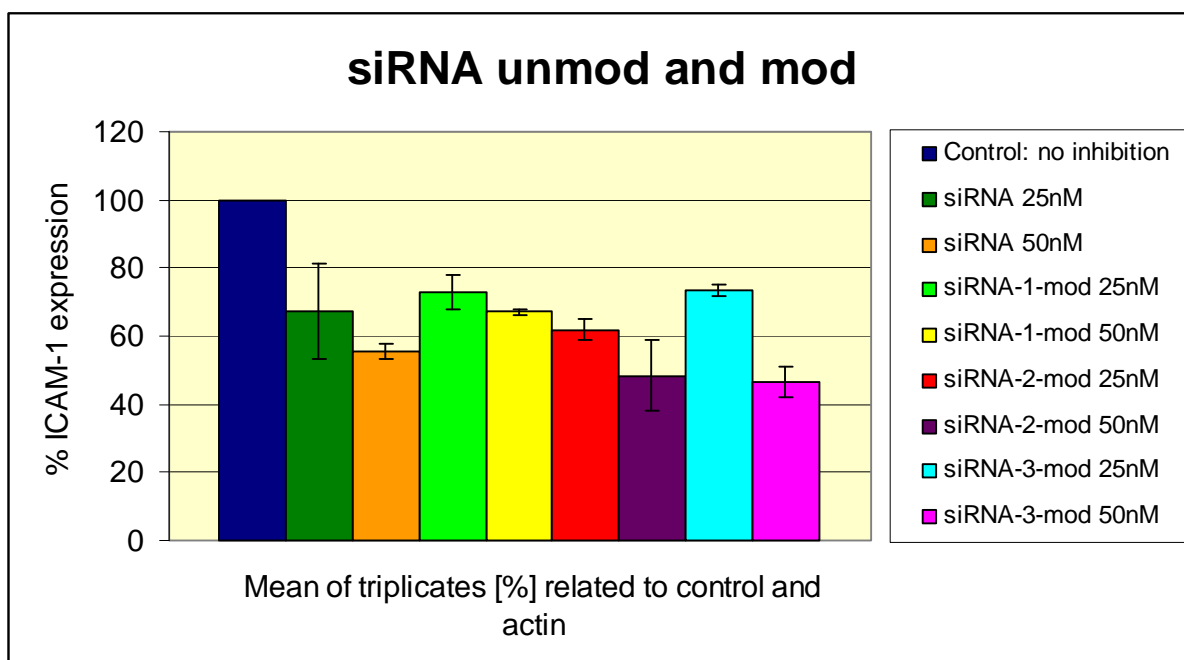
[Figure 28] Summary of concentrations: 25 and 50 nM (siRNA, siRNA 1U*, siRNA 2U* and siRNA 3U*).

When designing oligonucleotides with new modifications, such as the performed 2'-O-lysylaminohexyl uridine modification, an improvement of properties should be reflected in *in vitro* tests. In our study the alicaforsen sequence, which belongs to the first generation of oligonucleotides, was used as model sequence to test the mentioned modifications. It is a phosphorothioate referring to the exchange of oxygen atom for sulphur, which has been proven to inhance stability against enzymatic degradation. The main feature distinctive for the second generation is a modification at the 2'- position. Oligonucleotides with an alkoxy substituent in position 2' proved to be stable against DNA or RNA cleaving enzymes. This increase in stability has been explained by the steric hindrance, which protects loci usually attacked by nucleases at the phosphate groups. Caution has to be taken with the length of alkyl substituent as base pairing properties decrease with increasing length of the substituent. 2'-O-substitued oligonucleotides proved to be unable to catalyze enzymatic degradation of the complementary RNA by activation of RNAse H. Thus different combinations of modifications were tried with success. Oligonucleotides modified at both ends with at least five unmodified oligonucleotides between, proved to have the ability to activate RNAse H. The unmodified region was responsible for the activation of RNAse H, whereas the end modifications protected against enzymatic degradation. The inherence of 6-aminohexyl group, which is presented in the third generation of ASOs by zwitterionic antisense oligonucleotides,

86

at the 2' position not only increases stability against enzymatic degradation, but also minimizes the electrostatic repulsion of the sense and antisense strands leading to improved hybridization affinity.

In alicaforsen series the observed effect was dependent on the number of 2-O-lysylaminohexyl modifications. Undeniably modifications in the middle and at the end of a sequence had an ability to suppress gene expression much better than modifications done only in the middle of the sequence. These loci are the core sites where sequences target mRNA and inhibit synthesis of the aimed proteins. The first modification had a negative impact on the in vitro effect, but the substances with two and three modifications had higher efficiency than the unmodified phosphorothioate. It is essential to take into consideration the interference between the transfection reagent (lipofectamin 2000 was used in this study) and oligonucleotides, which can have an influence on target inhibition. Normally oligonucelotides form a complex with the cationic agents inhancing their uptake through the negatively charged cell membrane. It is obvious that the amount of reagent, the type of reagent and the complex-formation-properties influence the effectivity of the tested oligonucleotide. With regard to modified sequences this issue gets increasingly important, since the modified oligos are longer and their structure is more complex. The reason that one cationic side chain had an adverse effect on the *in vitro* activity can not be deduced from the available data. A lower transfection efficiency caused by lower liposomal uptake in the cationic liposomes formed by the covalently attached cationic groups is possible. More experiments with changing ratios of oligonucleotide: lipofectamine or cellular uptake studies with fluorescent oligonucleotide could help elucidate the cause of this finding. Concentrations 100 and 200 nM of applied DNA substances were chosen according to therapeutic applications in literature. The expected dose dependence was observed for the oligonucleotides with one and three 2'-O-lysylaminohexyl chains, while for the one with two modifications no significant difference was detected between 100 and 200 nM concentrations. Again, unexpected complexation disturbance may be a possible explanation, but irregularities in sample preparation or western blot quantification can contain possible error sources. Another important issue is the presence of impurities in oligonucleotides, which can have an effect on the inhibition. In this study all designed oligonucleotides were tested for their purity in the beginning of the experiment and all sequences showed to be pure.

siRNA is a young phenomenon and its results support the effect of alicaforsen in the inhibition of ICAM-1. With respect to the influence of the 2'-O-lysylaminohexyl groups, similar results were obtained for the tested siRNA sequence. Like for the antisense

oligonucleotide, the first incorporated lysylaminohexyl chain negatively influenced the *in vitro* target downregulation. While the second modified nucleotide raised the gene silencing activity to the same or higher level as the one found for the unmodified siRNA, the third modification did not significantly raise the activity further. It has to be pointed out that the third modified nucleotide was incorporated at the 5'-terminus of the antisense strand. It has been demonstrated that efficient siRNA sequences possess lower thermodynamic stability at the 5'-end of the antisense strand than at the other terminus (68). Since the cationic chain increases the thermodynamic stability, especially when incorporated at terminal sites of RNA oligonucleotides (data not published), lower strand bias by DICER and subsequent lowered activity is expected. Although stability against degrading enzymes (and possibly transfection efficiency) is increased by the third 2'-O-lysylaminohexyl nucleotide, this is abolished by the poorer incorporation in the molecular effector, DICER. In this experimental setting, a lower concentration of siRNA led to a similar extent of target down-regulation to alicaforsen. In the case of the modified siRNAs, all tested substances showed the expected dose dependence within a concentration range of 25-50 nM. The poor stability and very expensive synthesis of siRNA could be the main problems for the siRNA usage in the treatment of BBBs diseases in the future. Despite of these drawbacks siRNA could be a very beneficial in some of these illnesses.

All sequences showed good results. For future developments of the tested modification it depends on the toxicity of sequences, their stability, their efficient dose, complexity of their synthesis and economical aspects. All substances are promising for possible treatment in diseases of Central Nervous System such as Meningitis, Multiple Sclerosis, Neurological trypanosomiasis, Progressive multifocal leukoencephalopathy, De Vivo disease and others (see 2.1.4.).

7.   CONCLUSION

In this study it could be shown that 2'-O-lysylaminohexyl modification can be used in both oligonucleotides and siRNA. 2'-O-lysylaminohexyl modification is advantageous in better cellular uptake of oligonucleotides and siRNA, and in their better stability against enzymatic degradation. These properties have been reflected in a higher inhibition of the target protein ICAM-1 than is shown by unmodified sequences. The ICAM-1 target was used as a model to evaluate the influence of modifications. The number of modifications correlated with the *in vitro* effect on the inhibition. The effect of gene silencing was stronger with increasing number of modifications. Substances with two and three modifications had higher efficiency than the unmodified phosphorothioate or siRNA. Both modified ODN and siRNA were able to significantly reduce the levels of the target protein ICAM-1 (50%). The most efficient concentration for ODN was 200 nM, for siRNA 50 nM, so the concentration needed to achieve the best effect was lower for siRNA. The results determined that alicaforsen can effectively reduce ICAM-1 not only in gastrointestinal tract culture models, but in cells cultured in BBB conditions. Therefore all tested DNA and siRNA substances especially with two and three modifications are promising for treatment of CNS diseases.

8. LITERATURE

(1) Dietrich JB: The adhesion molecule ICAM-1 and its regulation in relation with the blood-brain barrier. *J Neuroimmunol* **128**(1-2):58-68, 2001.

(2) Abbott NJ: Dynamics of CNS barriers: evolution, differentiation, and modulation. *Cell Mol Neurobiol* **25**(1):5-23, 2005.

(3) Segal MB and Zlokovic BV: The blood-brain barrier, amino acids and peptides, Kluwer academic publishers group, Dordrecht, Boston, London, 1990.

(4) Huber JD, Egleton RD, and Davis TP: Molecular physiology and pathophysiology of tight junctions in the blood–brain barrier. *Trends Neurosci* **24**(12):719-725, 2001.

(5) Perry VH, Anthony DC, Bolton SJ, and Brown HC: The blood-brain barrier and the inflammatory response. *Mol Med Today* **3**(8):225-341, 1997.

(6) Beam TRJr and Allen JC: Blood, brain, and cerebrospinal fluid concentrations of several antibiotics in rabbits with intact and inflamed meninges. *Antimicrob Agents Chemother* **12**(6):710-6, 1977.

(7) Calabresi PA: Diagnosis and management of multiple sclerosis. *Am Fam Physcian* **70**(10):1935-1947, 2004.

(8) Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazullo JJ, and Krishna S: The trypanosomiases. *Lancet* **362**(9394):1469-80, 2003.

(9) Wyen C, Hoffmann C, Schmeisser N, Wohrmann A, Qurish N, Rockstroh J, Esser S, Rieke A, Ross B, Lorenzen T, Schmitz K, Stenzel W, Salzberger B, and Fatkenheuer G: Progressive multifocal leukencephalopathy in patients on highly active antiretroviral therapy: survival and risk factors of death. *J Acquir Immune Defic Syndr* **37**(2):1263-1268, 2004.

(10) Pascual JM, Wang D, Lecumberri B, Yang H, Mao X, Yang R, and De Vivo DC: GLUT1 deficiency and other glucose transporter diseases. *Eur J Endocrinol* **150**(5):627-33, 2004.

(11) Klepper J and Voit T: Facilitated glucose transporter protein type 1 (GLUT1) deficiency syndrome: impaired glucose transport into brain - a review. *Eur J Pediatr* **161**(6):295-304, 2002.

(12) Nagele RG: Alzheimer's disease: new mechanism for an old problem. *UMDNJ research* **7**(2), 2006.

(13) Abbott NJ, Roennbaeck L, and Hansson E: Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* **7**(1):41-53, 2006.

(14) Kapiotis S, Quehenberger P, Sengoelge G, Partan C, Eher R, Strobl H, Bevec D, Zapolska D, Schwarzinger I, and Speiser W: Modulation of pyrogen-induced upregulation of endothelial cell adhesion molecules (CAMs) by interleukin-4: transcriptional mechanisms and CAM-shedding. *Circ Shock* **43**(1):18-25, 1994.

(15) Frijns CJM and Kappelle CJ: Inflammatory cell adhesion molecules in ischemic cerebrovascular disease. *Stroke* **33**(8):2115-2122, 2002.

(16) Lee SJ and Benveniste EN: Adhesion molecule expression and regulation on cells of the central nervous system. *J Immunol* **98**(2):77-88, 1999.

(17) Rosette C, Roth RB, Oeth P, Braun A, Kammerer S, Ekblom J, and Denissenko M.F.: Role of ICAM-1 in invasion of human breast cancer cells. *Carcinogenesis* **26**(5):943-950, 2005.

(18) Chiang MY, Chan H, Zounes MA, Freier SM, Lima WF, and Bennett CF: Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two dinstrict mechanisms. *J Biol Chem* **266**(27):18162-18171, 1991.

(19) Yoshikawa T, Yoshida N, Manabe H, Terasawa Y, Takemura T, and Kondo M: α-tocopherol protects against expression of adhesion molecules on neutrophils and endothelial cells. *Biofactors* **7**(1-2):15-19, 1998.

(20) Combe C, Dupláa C, Couffinhal T, Moreau C, and Bonnet J: Induction of intercellular adhesion molecule-1 by monocyte adhesion to endothelial cells in human culture system. *J Cell Physiol* **164**(2):295-303, 1995.

(21) Reiss Y, Hoch G, Deutsch U, and Engelhardt B: T cell interaction with ICAM-1-deficient endothelium in vitro: essential role for ICAM-1 and ICAM-2 in transendothelial migration of T cells. *Eur J Immunol* **28**(10):3086-3099, 1998.

(22) Elovaara I, Ukkonen M, Leppakynnas M, Lehtimaki T, Luomala M, Peltola J, and Dastidar P.: Adhesion molecules in multiple sclerosis: relation to subtypes of disease and methylprednisolone therapy. *Arch Neurol* **57**(4):546-551, 2000.

(23) Woodmann SE, Benveniste EN, Nath A, and Berman JW: Human immunodeficiency virus type 1 TAT protein induces adhesion molecule expression in astrocytes. *J Neurovirol* **5**(6):678-684, 1999.

(24) Rieckmann P, Nunke K, Burchhardt M, Albrecht M, Wiltfang J, Ulrich M, and Felgenhauer K: Soluble intercellular adhesion molecule-1 in cerebrospinal fluid: an

indicator for the inflammatory impairment of the blood– cerebrospinal fluid barrier. *J Neuroimmunol* **47**(2):133-140, 1993.

(25) Crooke ST: Molecular mechanisms of action of antisense drugs. *Biochim Biophys Acta* **1489**(1):31-43, 1999.

(26) Noe CR and Kaufhold L: Chemistry of antisense oligonucleotides, in Methods and principles in medicinal chemistry, new trends in synthetic medicinal chemistry. Wiley-VCH: Weinheim, edited by Gualtieri F, 261-347, 2000.

(27) Wacheck V and Zangemeister-Wittke U: Antisense molecules for targeted cancer therapy. *Crit Rev Oncol Hemat* **59**(1):65-73, 2006.

(28) Mirabelli CK and Crooke ST: Antisense oligonucleotides in the context of modern molecular drug discovery and development, in Antisense research and application. Boca Raton, CRC press, edited by Crooke ST and Lebleu B, 7-36, 1993.

(29) Urban E and Noe C: Structural Modifications of Antisense Oligonucleotides. *Farmaco* **58**(3):243-58, 2003.

(30) Crooke ST: Basic principles of antisense technology, in Antisense drug technology: principles, strategies and applications, Marcel Dekker Inc, Basel, edited by Crooke ST, chapter 1, 2000.

(31) Donis-Keller H: Site specific enzymatic cleavage of RNA. *Nucleic Acids Res* **7**(1):179–192, 1979.

(32) Baker BF, Lot SS, Condon TP, Cheng-Flournoy S, Lesnik EA, Sasmor HM, and Bennett CF: 2'-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. *J Biol Chem* **272**(18):1994-2000, 1997.

(33) Sazani P, Kang S-H, Maier MA, Wei C, Dillman J, Summerton J, Manorahan M, and Kole R: Nuclear antisense effects of neutral, anionic and cationic oligonucleotide analogs. *Nucleic Acids Res* **29**(19):3965-3974, 2001.

(34) Winkler J, Urban E and Noe CR: Oligonucleotides conjugated to short lysine chains. *Bioconjug Chem* **16**(4):1038-44, 2005.

(35) Thein SL and Wallace RB: The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders, in Human genetic diseases: a practical approach, IRL Press, Oxford, edited by Davies KE, chapter 3, 33–50, 1986.

(36) Crooke PD: Medicinal chemistry of antisense oligonucleotides, in Antisense drug technology: principles, strategies and applications, Marcel Dekker Inc, Basel, edited by Crooke ST, chapter 2, 2000.

(37) www.isispharm.com

(38) Kurreck J: Antisense technologies improvement through novel chemical modifications. *Eur J Biochem* **270**(8):1628-1644, 2003.

(39) Manoharan M: 2'-carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. *Biochim Biophys Acta* **1489**(1):117-130, 1999.

(40) Monia BP, Lesnik EA, Gonzalez C, Lima WF, McGee D, Guinosso CJ, Kawasaki AM, Cook PD, and Freier SM: Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* **268**(19):14514-14522, 1993.

(41) Manoharan M, Prakash TP, Barber-Peoc'h I, Bhat B, Vasquez G, Ross BS, and Cook PD: N-(2-cyanoethoxycarbonyloxy)succinimide: A new reagent for protection of amino groups in oligonucleotides. *J Org Chem* **64**(17):6468-6472, 1999.

(42) Braasch DA and Corey DR: Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chem Biol* **8**(1):1-7, 2001.

(43) Braasch DA, Liu Y, and Corey DR: Antisense inhibition of gene expression in cells by oligonucleotides incorporating locked nucleic acids: effect of mRNA target sequence and chimera design. *Nucleic Acids Res* **30**(23):5160-5167, 2002.

(44) Winkler J, Urban E, Losert D, Wacheck V, Pehamberger H, and Noe CR: A novel concept for ligand attatchment to oligonucleotides via the 2'-succinyl linker. *Nucleic Acids Res* **32**(2):710-18, 2004.

(45) Tung C-H and Stein S: Preparation and Applications of Peptide-Oligonucleotide Conjugates. *Bioconjug Chem* **11**(5):605-618, 2000.

(46) Shanahan WR: ISIS 2302, an antisense inhibitor of intracellular adhesion molecule 1 (ICAM-1), in Antisense drug technology: principles, strategies and applications, Marcel Dekker Inc, Basel, edited by Crooke ST, chapter 29, 2000.

(47) Holmlund JT: Applying antisense technology: Affinitak and other antisense oligonucleotides in clinical development. *Ann N Y Acad Sci* **1002**:244-251, 2003.

(48) Glover JM, Leeds JM, Mant TG, Amin D, Kisner DL, Zuckerman JE, Geary RS, Levin AA, and Shanahan WRJr: Phase I Safety and Pharmacokinetic Profile of an Intercellular

Adhesion Molecule-1 Antisense Oligodeoxynucleotide (ISIS 2302). *J Pharmacol Exp Ther* **282**(3):1173-1180, 1997

(49) Paulus HE, Egger MJ, Ward JR, and Williams HJ: Analysis of improvement in individual rheumatoid arthritis patients treated with disease-modifying antirheumatic drugs, based on the findings in patients treated with placebo. *Arthritis Rheum* **33**(4):477-484, 1990.

(50) Mehta RC, Stecker KK, Cooper SR, Templin MV, Tsai YJ, Condon TP, Bennett CF, and Hardee GE: Intercellular Adhesion Molecule-1 Suppression in Skin by Topical Delivery of Anti-Sense Oligonucleotides. *J Invest Dermatol* **115**(5):805-812, 2000.

(51) Rao M and Sockanathan S: Molecular mechanisms of RNAi: implications for development and disease. *Birth defect Res C Embryo Today* **75**(1):28-42, 2005.

(52) www.nastech.com

(53) Opalinska JB and Gewirtz AM: Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov* **1**(7):503-517, 2002.

(54) Szweykowska-Kulinska Z, Jarmolowski A, and Figlerowicz M: RNA interference and its role in the regulation of eucaryotic gene expression. *Acta Biochim Pol* **50**(10):217-229, 2003.

(55) Cejka D, Losert D, and Wacheck V: Short interfering RNA (siRNA): tool or therapeutic? *Clin Sci* **110**(1):47-58, 2006.

(56) Aigner A: Applications of RNA interference: current state and prospects for siRNA based strategies in vivo. *Appl Microbiol Biotechnol* **76**(1):9-21, 2007.

(57) Kim VN: RNA interference in functional genomics and medicine. *J Korean Med Sci* **18**(3):309-318, 2003.

(58) www.nobelprize.org

(59) Paroo Z and Corey DR: Challenges for RNA in vivo. *Trends Biotechnol* **22**(8):390-394, 2004.

(60) Corey DR: Chemical modification: the key to clinical application of RNA interference? *J Clin Invest* **117**(12):3615-3622, 2007.

(61) Harborth J, Elbashir SM, Vandenburgh K, Manninga H, Scaringe SA, Weber K, and Tuschl T: Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev* **13**(2):83-105, 2003.

(62) Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Röhl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, and Vornlocher H-P: Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**(7014):173-178, 2004.

(63) Hall AH, Wan J, Shaughnessy EE, Ramsay Shaw B, and Alexander KA: RNA interference using boranophosphate siRNAs: structure-activity relationships. *Nucleic Acid Res* **32**(20):5991-6000, 2004.

(64) Swayze EE, Siwkowski AM, Wancewicz EV, Migawa MT, Wyrzykiewicz TK, Hung G, Monia BP, and Bennett CF: Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity. *Nucleic Acid Res* **35**(2):687-700, 2006.

(65) Allerson CR, Sioufi N, Jarres R, Prakash TP, Naik N, Berdeja A, Wanders L, Griffey RH, Swayze EE, and Bhat B: Fully 2'-modified oligonucleotides duplexes which improved in vitro potency and stability compared to unmodified small interfering RNA. *J Med Chem* **48**(4):901-904, 2005.

(66) Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, Johnson JM, Lim L, Karpilow J, Nichols K, Marshall W, Khvorova A, and Linsley PS: Position-specific chemical modifications of siRNA reduces "off-target" transcription silencing. *RNA* **12**(7):1197-1205, 2006.

(67) Dowler T, Bergeron D, Tedeschi AL, Paquet L, Ferrari N, Damha MJ: Improvements in siRNA properties mediated by 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (FANA). *Nucleic Acids Res* **34**(6):1699-1675, 2006.Ginobbi P, Geiser TA, Ombres D, and Citro G: Folic acid-polylysine carrier improves efficacy of c-myc antisense oligodeoxynucleotides on human melanoma (M14) cells. *Anticancer Res* **17**(1A):29-35, 1997.

(68) Dande P, Prakash TP, Sioufi N, Gaus H, Jarres R, Berdeja A, Swayze EE, Griffey RH, and Bhat B: Improving RNA interference in mammalian cells by 4'-thio modified small interfering RNA (siRNA): effect on siRNA activity and nuclease stability when used in combination with 2'-O-alkyl modifications. *J Med Chem* **49**(5):1624-1634, 2006.

(69) Watts JK, Choubdar N, Sadalapure K, Robert F, Wahba AS, Pelletier J, Pinto BM, and Damha MJ: 2'-fluoro-4'-thioarabino-modified oligonucleotides: conformational switches linked to siRNA activity. *Nucleic Acid Res* **35**(5):1441-51, 2007.

(70) Muratovska A and Eccles MR: Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett* **558**(1-3):63-68, 2004.

(71) Kim SH, Jeong JH, Lee SH, Kim SW, and Park TG: PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. *J Control Release* **116**(2):123-129, 2006.

(72) McNamara JO, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, and Giangrande PH: Cell type-specific delivery of siRNAs with aptamer siRNA chimeras. *Nat Biotechnol* **24**(8):1014-1015, 2006.

(73) Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, Feng Y, Palliser D, Weiner DB, Shankar P, Marasco WA, and Lieberman J: Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* **23**(6):709-717, 2005.

(74) Pushparaj PN and Melendez AJ: Short interfering RNA (siRNA) as a novel therapeutic. *Clin Exp Pharmacol Physiol* **33**(5-6):504-510, 2006.

(75) www.sirna.com

(76) www.acuitypharma.com and www.opko.com

(77) Corey DR: RNA learns from antisense. *Nat Chem Biol* **3**(1):8-11, 2007.

(78) www.nih.gov

(79) www.asuragen.com

(80) Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY, Ansari AM, Scaria PV, Woodle MC, Lu P, and Rouse BT: Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor-pathway genes: Therapeutic strategy for herpetic stromal keratitis. *Am J Pathol* **165**(6):2177-2185, 2004 .

(81) Kao SC, Krichevsky A, Kosik K, and Tsai L-H: BACE1 suppression by RNA interference in primary cortical neurons. *J Biol Chem* **279**(3):1942-1949, 2003.

(82) Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, and Lieberman J.: RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* **9**(3):347-351, 2003.

(83) McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, and Kay MA: RNA interference in adult mice. *Nature* **418**(6893):38-9, 2002.

(84) Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, and Rossi J: Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* **20**(5):500-505, 2002.

(85) Zheng BJ, Guan Y, Tang Q, Du C, Xie FY, He ML, Chan KW, Wong KL, Lader E, Woodle MC, Lu PY, Li B, and Zhong N: Prophylactic and therapeutic effects of small interfering RNA targeting SARS-coronavirus. *Antivir Ther* **9**(3):365-374, 2004.

(86) Ralph GS, Radcliffe PA, Day DM, Carthy JM, Leroux MA, Lee DC, Wong LF, Bilsland LG, Greensmith L, Kingsman SM, Mitrophanous KA, Mazarakis ND, and Azzouz M.: Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med* **11**(4):429-433, 2005.

(87) Hadj-Slimane R, Lepelletier Y, Lopez N, Garbay C, and Raynaud F: Short interfering RNA (siRNA), a novel therapeutic tool acting on angiogenesis. *Biochimie* **89**(10):1234-1244, 2007.

(88) Weiss TL, Selleck SE, Reusch M, and Wintroub BU.: Serial subculture and relative transport of human endothelial cells in serum-free, defined conditions. *In Vitro Cell Dev Biol* **26**(8):759-768, 1990.

(89) Hughes S.E.: Functional characterization of the spontaneously transformed human umbilical vein endothelial cell line ECV304: use in an vitro model of angiogenesis. *Exp Cell Res* **255**(1):171-185, 1996.

(90) Takahashi K, Sawasaki Y, Hata JI, Mukai K, and Goto T: Spontaneous transformation and immortalization of human endothelial cells. *In Vitro Cell Dev Biol* **26**(3 Pt 1):265-274, 1990.

(91) Kiessling F, Kartenbeck J, and Haller C: Cell-cell contacts in the human cell line ECV304 exhibit both endothelial and epithelial characteristics. *Cell Tissue Res* **297**(1):131-140, 1999.

(92) Hurst RD and Fritz IB: Properties of an immortalized vascular endothelial/glioma cell co-culture model of the blood brain-barrier. *J Cell Physiol* **167**(1):81-99, 1996.

(93) Patzel V., Steidl U., Kronenwett R., Haas R., and Sczakiel G.: A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability. *Nucleic Acids Res* **27**(22):4328-4334, 1999.

(94) Leonetti JP, Degols G, and Lebleu B: Biological activity of oligonucleotide-poly (L-lysine) conjugates: mechanism of cell uptake. *Bioconjug Chem* **1**(2):149-153, 1990.

(95) Degols G, Leonetti JP, Benkirane M, Devaux C, and Lebleu B: Poly (L-lysine) conjugated oligonucleotides promote sequence specific inhibition of acute HIV-1 infection. *Antisense Res Dev* **2**(4):293-301, 1992.

(96) Clarenc JP, Degols G, Leonetti JP, Milhaud P, and Lebleu B: Delivery of antisense oligonucleotides by poly (L-lysine) conjugation and liposome encapsulation. *Anticancer Drug Des* **8**(1):81-94, 1993.

(97) Ginobbi P, Geiser TA, Ombres D, and Citro G: Folic acid-polylysine carrier improves efficacy of c-myc antisense oligodeoxynucleotides on human melanoma (M14) cells. *Anticancer Res* **17**(1A):29-35, 1997.

(98) Nishiwaki Y, Yokota T, Hiraoka M, Miyagishi M, Taira K, Isobe M, Mizusawa H., and Yoshida M.: Introduction of short interfering RNA to silence endogenous E-selectin in vascular endothelium leads to successful inhibition of leukocyte adhesion. *Biochem Biophys Res Comm* **310**(4):1062-1066, 2003.

(99) Walker T, Wendel HP, Tetzloff L, Heidenreich O, and Ziemer G: Suppression of ICAM-1 in human venous endothelial cells by small interfering RNAs. *Eur J Cardiothorac Surg* **28**(6):816-820, 2005.

(100) Khvorova A, Reynolds A, and Jayasena SD: Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**(2):209-216, 2003.

9. SUMMARY

Conjugation of ligands to antisense oligonucleotides is a promising approach for enhancing their effects on gene expression. In this study 2'-O-lysylaminohexyl group was linked to the uridine base, which replaces one, two or three thymine bases thus modifies the oligonucleotides. This exchange of bases was tested for improvement of silencing target protein expression.

Effectivity of modifications in silencing target protein expression was examined with the alicaforsen sequence (DNA) and siRNA. Alicaforsen, currently in clinical trial 3, is a phosphorothioate targeting ICAM-1, which was the model used to evaluate the influence of modifications. The same target was chosen for siRNA to compare the efficiency of DNA and siRNA substances. For the first time, down-regulation of ICAM-1 was shown on the blood brain barrier cell line ECV304.

Unmodified/modified antisense oligonucleotides and siRNA sequences were transfected into ECV304 cells with the help of a transfection agent lipofectamine 2000. After 24 hours of transfection cells were disrupted by a chemical lysis. Protein concentrations were determined by Bradford protein assay. ICAM-1 inhibition was assessed with western blot. The inhibitory effect of ICAM-1 was normalized to the corresponding actin and untreated cells. ICAM-1 protein levels were quantified by a densitometry of autoradiograms with a densitometer and a software program Quantity One. All experiments were conducted at least in triplicates.

2'-O-lysylaminohexyl modification improved the cellular uptake of oligonucleotides and siRNA, and their better stability against degradation by nucleases. Both modified DNA and siRNA sequences have shown inhibition of the target protein ICAM-1 by 50%. The increasing number of modifications (one up to three) influenced the effect of gene expression. Sequences with two and three modifications had higher efficiency than the unmodified oligonucleotides or siRNAs. The most efficient concentration for gene silencing was 200 nM by oligonucleotides and 50 nM by siRNA.

2'-O-lysylaminohexyluridine could be shown to be a beneficiary modification of DNA and siRNA used for gene silencing studies. All tested sequences, especially with two and three modifications are promising for treatment of CNS diseases.

10.   SOUHRN

V této studii se projevuje nadějná možnost zvýšení genové exprese konjugací ligandů na antisense oligonukleotidy a siRNA. Při modifikaci se využilo 2'-O-lysylaminohexylové skupiny vázané na uridinovou bazi, která nahradila jednu, dvě až tři baze thyminové. Uvedené záměny a modifikace bazí byly testovány pro zlepšení inhibice proteinové exprese.

Efektivita modifikací v inhibici proteinové exprese byla zkoušena na alicaforsenové sekvenci (DNA) a siRNA. Alicaforsen, který se nachází ve třetí fázi klinické studie, je phosphorothioát komplementární k ICAM-1, siRNA, jenž nově prokázala afinitu k ICAM-1, čehož bylo využito při porovnání účinnosti výše zmíněných modifikací a objevení následných možností uplatnění alicaforsen a siRNA v terapii. Poprvé byla pozorována a prokázána down-regulace ICAM-1 na buněčné linii ECV304, která imituje prostředí hematoencefalické bariéry.

Nemodifikované/modifikované sekvence oligonukleotidů a siRNA byly transfekovány do ECV304 buněk. Po 24 hodinové transfekci byly buňky rozrušeny chemickou lýzou. Koncentrace získaných proteinů byly stanoveny Bradford analýzou a následná inhibice ICAM-1 byla vyhodnocena western blotem. Hodnoty inhibicí byly standardizovány k odpovídajícímu aktinu a kontrolním buňkám. Vyhodnocení genové exprese proběhlo pomocí denzitometrie autoradiogramů a počítačového programu Quantity One. Všechny pokusy byly provedeny nejméně třikrát.

Modifikace 2'-O-lysylaminohexylem zlepšila uptake oligonukleotidů buněčnou membránou a zároveň zvýšila jejich rezistenci vůči nukleázám. Modifikované DNA i siRNA potlačily genovou expresi ICAM-1 přibližně 50%. Zvýšením počtu modifikací (z jedné na dvě až tři) se zvýšila inhibice ICAM-1 oproti nemodifikovaným oligonukleotidům a siRNA sekvencím. Koncentrace oligonukleotidů, která prokázala nejvyšší inhibici, byla 200 nM u DNA a 50 nM u siRNA.

2'-O-lysylaminohexyluridin by mohla být úspěšnou modifikací DNA a siRNA pro studie genových inhibicí. Všechny testované sekvence oligonukleotidů, zejména se dvěma a třemi modifikacemi, se ukázaly být slibnými pro léčbu chorob CNS.

Výsledky této práce byly publikovány v: Winkler J, Gilbert M., Kocourková A, Stessl M, and Noe CR: 2'-O-lysylaminohexyl oligonucleotides: modifications for antisense and siRNA, *ChemMedChem* **3**(1):102-110, 2008.