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Key Note Address

New Insight and Specific Aspects of the Structure-Function Relationship of Antisense Oligonucleotides and RNA Interference

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Meanwhile a remarkable number of different classes of oligomeric nucleic acids serve as promising lead compounds for drug development. They are derived from molecular biology and act according to new mechanisms that clearly extend the classical pharmaceutical repertoire of modes of action and drug targets. While their mechanism of action is different most of them share similarities regarding their chemical synthesis, the delivery to target tissues and cells, and toxicology. The most advanced candidates of oligomeric nucleic acid drugs include antisense oligonucleotides and cytidine-phospho-guanosine (CpG) immunostimulatory oligonucleotides. In the laboratory, the development of biologically highly active double-stranded RNA (siRNA) as a drug proceeds rapidly.

Antisense nucleic acids, RNAi (RNA interference) and other classes of drugs that are directed against RNA as a target act sequence-specifically and it is reasonable to assume that recognition between the drug and the target involves base-specific interactions. Since long-chain target RNA is structured this implies that local target structure might influence apparent accessibility and recognition, thereby affecting the biological activity of the drug. New insights and specific aspects of the structure-function relationship of antisense oligonucleotides and RNAi will be presented and discussed.

The application of oligomeric nucleic acid drugs in vivo is limited by the low efficiency of cellular uptake and the lack of appropriate carrier systems. Here a new concept for the delivery of naked nucleic acid drugs to target cells and tissues via specific nucleotide sequence elements in cis will be discussed.

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Technology

Retrieval of Cholinergic Balance by Antisense Oligonucleotides

From animal models to clinical trials

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Recently accumulated evidence demonstrates frequent disease-related changes in the alternative splicing patterns of gene products controlling numerous physiological functions [1]. The efficacy of antisense targeting of one of the several splicing variants of such mammalian genes was tested for acetylcholinesterase (AChE). The enzyme, AChE, is intimately involved in cholinergic neurotransmission [2]. Alternative splicing induces, under conditions of cholinergic imbalance, overproduction of the rare "readthrough" variant AChE-R mRNA [3]. In mice, this is associated with electrophysiological hyperactivity [4], impaired working memory [5], hypersensitivity to head injury [6], prolonged contextual fear response [7] and weakened muscles [8]. All of these are transiently alleviated by murine (m)EN101, an antisense oligonucleotide that selectively induces the destruction of mouse AChE-R mRNA [9]. The pathophysiological relevance of AChE-R accumulation to neuromuscular functioning was evident from the accumulation of serum AChE-R in patients with myasthenia gravis (MG) and rats with experimental autoimmune MG (EAMG), both neuromuscular junction diseases with depleted acetylcholine receptors. In EAMG, we alleviated electromyographic abnormalities by nanomolar doses of rat (r)EN101, shown to selectively lower AChE-R in blood and muscle, yet leave unaffected the synaptic variant, AChE-S. While animals treated with placebo or conventional cholinesterase inhibitors continued to deteriorate, a 4-week daily oral administration of rEN101 improved survival, neuromuscular strength and clinical status in moribund EAMG rats [10]. Cynomolgus monkeys maintained normal health and locomotion activity during one week of oral or intravenous administration of human (h)EN101, which reduced AChE-R mRNA levels in spinal cord neurons and suppressed the cholinergic input into motoneurons. Finally, in clinical trials, human MG patient volunteers responded to one week oral hEN101 administration with conspicuous improvement in muscle functioning. These multi-species findings highlight the advantages of mRNA-targeted therapeutics for retrieving normal patterns of alternative splicing and achieving physiological homeostasis.

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Antisense Oligonucleotides – Mechanism, Chemistry and Therapeutics

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Since the discovery in the late 1970s that oligonucleotides could downregulate gene expression, antisense-technology has advanced to a new, powerful method of a rational drug design. In the meantime besides antisense-technology different modes or mechanisms of action of synthetic oligonucleotides are under investigation, such as e.g. ribozymes, aptamers, spiegelmers, and immunostimulatory CpG (cytidine-phospho-guanosine) oligonucleotides. 1998 the world's first commercial antisense drug Vitravene™ for treatment of cytomegalovirus (CMV) infections, was approved by the FDA.

Currently there are two antisense therapeutics under development in phase III clinical trials, ISISs 3521 (ISIS/Eli Lilly) for treatment of cancer as a selective inhibitor of protein kinase C-alpha, and Gentas G3139 (Genta/Aventis), a potent inhibitor of Bcl-2 expression. Genasense enhances the effectiveness of chemo-therapeutics e.g. Taxotere in patients with hematologic cancers and solid tumors.

In the last years a multitude of novel oligonucleotide modifications have been created and investigated to improve the properties of oligonucleotides compared to the uniformly phosphorothioate-modified oligonucleotides of the first generation. Examples will show how modifications e.g. in the phosphate backbone, the heterocyclic bases or the carbohydrate moiety influence stability, cellular uptake, toxicity and bioavailability.

Therapeutic Activity of Oligonucleotides in Murine Tumor Models

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Oligonucleotides (ON) containing CpG (cytidine-phospho-guanosine) dinucleotides within specific sequence contexts (CpG motifs) recently have been identified as danger signals activating rodent or primate immune cells triggering release of th1 cytokines. A variety of antisense phosphorothioate ON developed to target factors potentially involved in tumor growth and/or apoptosis suppression demonstrated antitumor activity in mice models. In particular, the compound G3139 (Genasense™) targeting BCL-2 is currently tested in phase III clinical trials on various tumors in combination therapy. Since G3139 harbors CpG motifs we questioned whether the effects seen in animal models might also be achieved by a phosphorothioate ON just optimized for immune activation in rodent cells. Therefore, the compound H1826 described recently by Hartmann et al. [1] was compared with G3139. To further elucidate the contribution of immune stimulation we also tested a G3139 derivative (G3139-5mC) methylated at C-5 of cytosine. Remarkably, H1826 or G3139 produced similar effects on xenografts (SCLC H69, ovarian carcinoma A2780) at doses of 1 mg/kg (H1826), or 12 mg/kg (G3139) per day administered i.v. via tail vein. In contrast, the methylated derivative of G3139 (at 12 mg/kg) showed no significant antitumor effect. G3139 at the doses applied induced strong enlargement of spleens. Moreover, H1826 and G3139 were tested at the syngeneic B16BL-6 melanoma lung metastasis model. Here, H1826 showed effects comparable to the anticancer compound dacarbazine while G3139 produced only moderate antimetastatic efficacy at the brink of significance. Immunostimulation was tested in vitro by flow cytometry of the activation markers CD80 and CD86 on CD19 positive murine splenocytes. Expression of activation markers essentially mirrored the effects observed in vivo. In particular, G3139-5mC was com-

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pletely inactive in vitro as well. Our data indicate a significant contribution of immunostimulatory components to antitumor effects in nude mice xenograft or syngeneic murine tumor models, and point to the need of using either immunostimulatory unresponsive in vivo models or immunostimulatory inactive antisense oligonucleotides for in vivo target validation and proof of concept for the antisense oligonucleotide drug format. Immunostimulatory active oligonucleotides, though, present an attractive therapeutic potential independent from target gene RNA sequences.

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Production of GMP Nucleic Acids

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Interest in commercial scale manufacturing of oligonucleotides is growing steadily based on the progression of several clinical candidates to the later stages of development as well as a growing number of clinical candidates entering Phase I clinical trials. Servicing this market need requires an integrated approach of the development of this capability from analytical method development to long term project management. A diverse pool of people resources (from chemistry to regulatory) and a significant investment of capital are required to establish this capability.

Contract manufacturing also means to explore the power of partnerships in developing and offering solutions that can significantly improve the quality and productivity of customers' research and manufacturing processes.

This presentation will focus on Eurogentec's approach and experience in meeting the needs of the customer and in integrating technologies and know-how into unique solutions which fit their customers' problems, help them speed products to market, and provide them with a competitive advantage.

Specific Silencing of HIV-1 Infection in Human T Cells by RNA Interference

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The RNA interference (RNAi) phenomenon is a recently observed process in which the introduction of a double stranded RNA (dsRNA) into a cell causes specific degradation of an mRNA containing the same sequence. To study dsRNA-mediated gene interference in HIV-1 infected cells, we have designed six longer dsRNA containing the HIV-1 gag and env genes.

Sense (ss) RNA were co-transfected with pNL4–3 into COS cells, and were also transfected into HIV-1 NL4–3 infected HeLa cells using cationic liposomes. The virus production in the culture supernatant was assessed by the HIV-1 p24 antigen assay. The HIV-1 mRNA levels were determined by on RT-PCR assay to identify the contribution of the dsRNA-mediated specific RNA interference.

The six longer dsRNAs containing the HIV-1 gag (G1–G3) and env (E1–E3) gene sequences reduced the p24 expression by about 95 % as compared to the untreated

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COS cells. The three dsRNA targeted to the HIV-1 env gene (E1–E3) effectively inhibited p24 expression was suppressed 20 to 24 fold by the dsRNAs (E1–E3), and 8 to 140 fold by the asRNA (E1–E3). The targeted inhibitory effects (96 %) were detected with the 531bp (7070~7600) E2-dsRNA, containing a major CD4 binding domain sequence of gp120 as the target of the HIV-1 env gene. In the infected HeLa CD4 cells, viral replication was inhibited 70 fold by E2-dsRNA, and 50-fold by the E2-asRNAs. Furthermore, dsRNA significantly suppressed HIV-1 infection in HeLa CD4 cells and PBMCs for a relatively long period of time. We demonstrated that Dicer localizes into HeLa CD4 and COS cells.

We also describe siRNA significantly suppressed HIV-1 infection in human T cells.

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RNA Interference in Leukemias and Lymphomas

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Leukemias are often characterized by nonrandom chromosomal translocations that, at the molecular level, induce the activation of specific oncogenes or create novel chimeric genes. They have frequently been regarded as optimal targets for gene-silencing approaches because of the large body of evidence that these single abnormalities directly initiate and maintain the malignant process. Despite the enormous therapeutic potential, gene-silencing approaches, e.g. by antisense therapy, are far from being fully implemented into the clinical setting and many clinical trials have revealed a rather disappointing effectiveness. Some of these difficulties stem from the inability to achieve sufficient power and sequence specificity of the gene-silencing strategy.

In our work, we focussed on the effective inhibition of fusion genes generated by chromosomal translocation involving small interfering RNAs. We demonstrate the inhibition of M-BCR/ABL in the CML cell line K562, which expresses the 210 kD variant of the BCR/ABL oncoprotein. The analysis of M-BCR/ABL after transfection of this siRNA in K562 cells was done by quantitative real-time RT-PCR and revealed a strong inhibitory effect of the M-BCR/ABL-specific siRNA. The M-BCR/ABL protein was also silenced to a barely visible level. The specificity of the siRNA was tested by comparison with a second siRNA, which was also targeted against the M-BCR/ABL fusion site but exhibited two point mutations within its central region. This siRNA was clearly less efficient in reduction of M-BCR/ABL. Downregulation of M-BCR/ABL was accompanied by the induction of apoptosis in the Ph+-K562 cell line after application of M-BCR/ABL-specific siRNAs. When compared to the induction of apoptosis after treatment with the "gold-standard" for BCR/ABL-positive leukemias, the ABL tyrosine-kinase inhibitor STI 571, the rate of cell killing was nearly the same for both approaches. However, an additive effect of combining the STI 571 and M-BCR/ABL siRNAs was not seen, possibly because of the high concentration of STI 571 (1 μmol/l) used in our studies. In the light of the increasing evidence that tumor cells rapidly develop a resistance to the tyrosine kinase inhibitor STI 571, either by a single point mutation within the ABL kinase domain or a variety of other cellular mechanisms bypassing the BCR/ABL pathway, a combined molecular therapy with low-molecular-weight drugs and RNA therapeutics would be a strategy worth testing.

With respect to lymphoma, the translocation t(2;5) occurs in up to 75 % of all pediatric cases of anaplastic large cell lymphoma (ALCL). The rearrangement combines the nucleophosmin protein NPM1 with the tyrosine-kinase gene ALK. The fusion-protein exhibits constitutive activation of the tyrosine-kinase ALK, leading to cellular transformation through activation of a variety of pathways involved in proliferation and cell survival. We achieved also a remarkable downregulation of NPM/ALK, as assessed by immunohistochemistry, when HeLa cells were co-transfected with an NPM/ALK expression vector and siRNAs covering the NPM/ALK fusion site.

In summary, the work so far done on siRNA-mediated downregulation of fusion genes in leukemias and lymphomas outlined the power of this strategy. A major

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drawback of antisense-based strategies, namely that the sequence specificity of the molecules used was often questionable, seems to be overcome with siRNAs.

Moreover, with help of the Withehead Institute's siRNA selection tool (http://jura.wi.mit.edu/bioc/siRNA/home.php) I suggest suitable siRNAs for a couple of additional chromosomal fusion sites. All sequences were carefully analysed by Blast search to exclude interaction of the siRNA with additional human genes.

These siRNA sequences are specific for some of the most frequent chromosomal fusion genes that may be of help for those who want a quick start on a particular chromosomal translocation.

RNA Synthesis, Purification and Analysis

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An optimized solid-phase synthesis of RNA using 2'-O-tert.-butyldimethylsilyl protected ribonucleoside phosphoramidites will be described, with particular attention given to the fine details. Deprotection conditions have been developed that result in higher yields of product with reduced risk of degradation during the various stages of deprotection. Subsequent purification of the crude RNA by anion-exchange and/or reversed phase HPLC is used to obtain the purities required for exacting applications such as NMR spectroscopy, X-ray crystallography and in vivo RNA interference experiments. Which purification methods are chosen depends upon the length of the RNA as well as the purity required. Analytical tools used to control the final purity and integrity of the RNA are analytical HPLC on high resolution anion-exchange and reversed phase columns plus an accurate mass determination by mass spectroscopy. The presentation will be illustrated with examples ranging from simple short RNAs with no structure to long RNAs with complex secondary and tertiary structures. The optimized methods described here have been used to obtain 10–20 mg amounts of RNA molecules as long as 56 mers.

Control of Gene Expression by Peptide Nucleic Acids (PNA)

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The pseudopeptide DNA mimic PNA (peptide nucleic acid) hybridizes efficiently and with high sequence discrimination to complementary RNA. When targeted to the translation initiation region, the 5'-untranslated region or to exon/intron junctions of mRNA it efficiently inhibits translation in an RNaseH independent reaction. PNA can also bind with high affinity to double stranded DNA by helix invasion, and thereby modulate gene expression at the transcriptional level. Furthermore, PNA has high biostability and is easy to chemically synthesize and modify. In the process of evaluating the prospects of developing PNA derived gene therapeutic anti-cancer drugs, we are studying PNA targeting of the oncogene mdm2. We have identified PNA oligomers that with cationic liposomal delivery down-regulate the expression of mdm2 protein, and also result in

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increased level of p53. Furthermore, the PNAs induce up-regulation of genes under control of the p53 response element. Finally these PNAs are toxic to jar cells acting in synergy with the anticancer drug camptothecin.

Results relating the peptide mediated cellular delivery of PNA antisense agents as well as their pharmacokinetic behavior in mice will also be presented.

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Clinical Development I: CpG and Antisense

Oligonucleotide Therapeutics and the Immune System

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In 1890, the New York surgeon William B. Coley learned of a case of spontaneous regression of metastatic sarcoma following infection of the tumor margin. More than a hundred years later it was demonstrated that bacterial DNA is responsible for the anti-tumor activity of bacterial extracts. Unmethylated CpG (cytidine-phospho-guanosine) dinucleotides in particular sequence contexts ("CpG motifs") are responsible for the immunostimulatory effects of bacterial DNA. Synthetic oligonucleotides (ODN) containing these sequences mimick bacterial DNA. ODN with potent human CpG motifs are excellent vaccine adjuvants in primates and humans. The vertebrate immune system has established toll-like receptor 9 (TLR9) to detect microbial DNA based on unmethylated CG dinucleotides within certain sequence contexts (CpG motifs). In humans, the expression of TLR9 is restricted to B cells and plasmacytoid dendritic cells (PDC). The PDC is characterized by the ability of rapidly synthesizing large amounts of type I IFN (IFN-a and IFN-b) in response to viral infection. In contrast to other dendritic cell subsets which express a broad profile of TLRs, the TLR profile in PDC is restricted to TLR7 and TLR9. So far, CpG DNA is the only defined microbial molecule recognized by PDC, while other microbial molecules such as LPS or poly I:C, due to the lack of the corresponding TLRs, do not stimulate PDC. An intriguing feature of PDC is its ability to simultaneously produce the two major Th1-inducing cytokines in humans, IFN-a and IL-12, both at high levels. The ratio of IFN-a versus IL-12 and the quantity of these cytokines are regulated by T helper cell-mediated co-stimulation via CD40 ligation. The ratio also depends on the differentiation stage of the PDC at the time of stimulation and the type of CpG ODN used. We propose a model in which the PDC functions as a switchboard for regulating Th1 versus Th2/Th0 responses: in the presence of appropriate microbial stimulation (such as CpG DNA), PDC trigger a Th1 response; in the absence of appropriate microbial stimulation PDC promote an unbiased T helper cell response (Th0) or Th2. First animal studies provide evidence that large murine tumors can be cured by CpG ODN and dendritic cells. In conclusion, CpG ODN represent a unique microbial stimulus for selective stimulation of PDC and B cells. Three different classes of CpG ODN with distinct functional profiles were established in our laboratory: CpG-A, CpG-B and CpG-C. These different classes of CpG ODN are promising candidates for the treatment of infectious disease, allergy and cancer.

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The Use of CpG Oligonucleotides for Immunotherapy of Cancer

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1. Background

Spontaneous regression of tumors has been observed for more than a century in patients who developed a bacterial infection in close proximity to a tumor. Unmethylated cytidiene-guanosine (CG) dinucleotides within particular base contexts (CpG motifs) have been identified to be responsible for the immunostimulatory effects. The identification of CpG (cytidine-phospho-guanosine) motifs allowed the development of CpG motif-containing oligodeoxynucleotides (CpG ODN) which mimic bacterial DNA. On the other side, it has been shown that dendritic cells (DC), manipulated in vitro, act as potent inducers of tumor immunity in murine tumor models. We decided to test the therapeutic potential of DC and CpG ODN in a C26 and a Renca Balb/c tumor model.

2. Methods

Syngeneic C26 colon carcinoma and Renca kidney cancer cell lines were used in a Balb/c tumor model. For tumor induction, 2×10^5 tumor cells were injected s. c. into one flank, or the same amount of cells per flank for the generation of two tumors (double the tumor mass at start of therapy). 100 µg CpG ODN 1826 was injected weekly starting on day 5 after tumor induction. Tumor size (length x width in mm^2) was measured three times weekly. Animals were killed when the tumor size exceeded 400 mm^2.

For the generation of dendritic cells (DC) bone marrow from mouse tibia and femur were depleted of T and B lymphocytes and granulocytes by magnetic beads. Unbound cells were cultured in medium supplemented with murine granulocyte macrophages colony-stimulating factor (mGM-CSF; 200 U/ml) and interleukin-4 (IL-4; 20 ng/ml). At day 7, irradiated tumor cells were added in a DC to tumor cell ratio of 5 to 1. For additional DC stimulation, CpG ODN 1826 (6 µg/ml) was added for the last 2 days. After 10 days loosely adherent cells were harvested, and expression of MHC II, CD80, CD86 and Ly6G was quantified by flow cytometry. For chemotherapy 20 mg/kg body weight of 5-fluorouracil were intraperitoneally coinjected with 100 mg/kg body weight of leucovorin.

3. Results

3.1. The anti-tumor activity of CpG ODN depends on the side of injection

Starting five days after C26 tumor challenge, weekly injections of CpG ODN were performed either into the

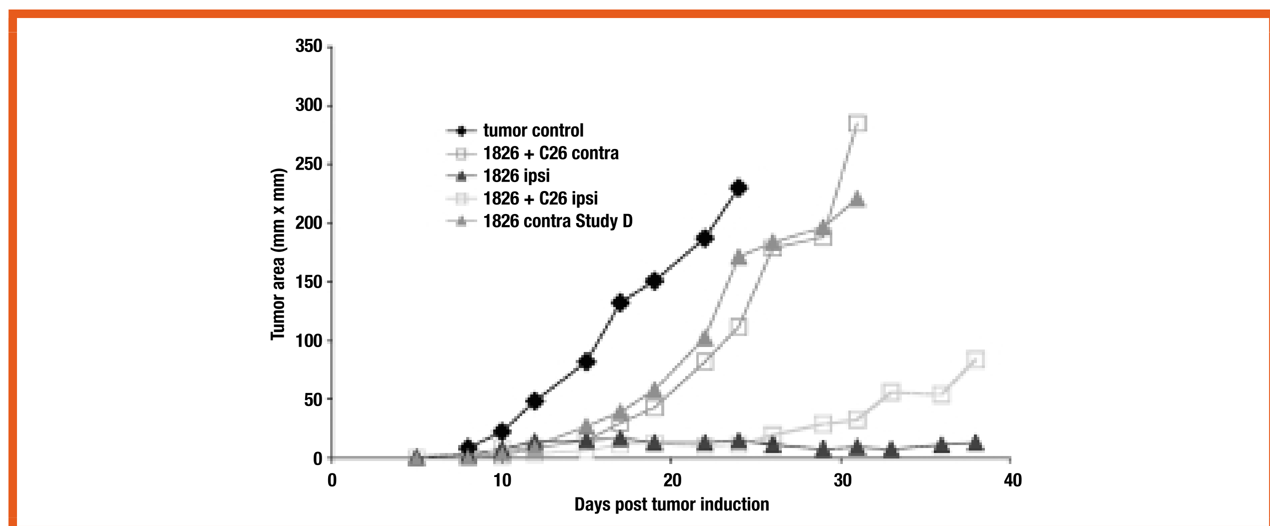

Fig. 1: Mice were challenged with tumor on day 0. Beginning on day 5, weekly injections of CpG ODN 1826 with and without irradiated C26 cells were performed. Tumor growth was monitored in untreated mice (n = 19), in mice with peritumoral injections of CpG ODN 1826 (n = 20), with peritumoral injections of CpG ODN 1826 coinjected with irradiated C26 cells (n = 7), in mice with injection of CpG ODN 1826 into the opposite flank (n = 6) or with coinjection of CpG ODN 1826 and irradiated tumor cells into the opposite flank (n = 7). The mean tumor size (mm^2) is depicted until the end of therapy on day 38 or until the first tumor within a group exceeded 400 mm^2.

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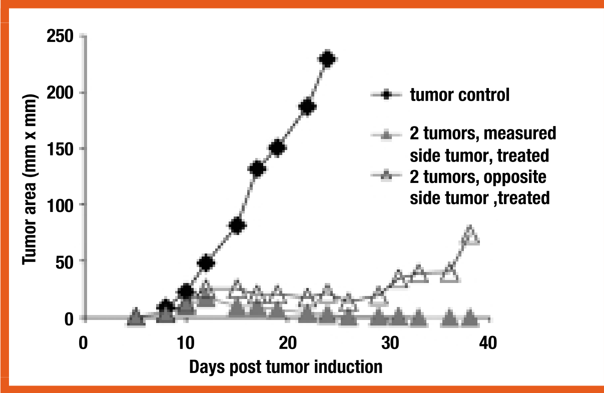

Fig. 2A: Mice were challenged with tumor on both flanks and CpG ODN 1826 was injected into the right flank (n=16) or left untreated (n=19). The growth of the tumor on the right flank and on the left flank was monitored separately. Tumor growth of the left tumor was significantly reduced in comparison to mice with CpG ODN 1826 alone injected contralaterally.

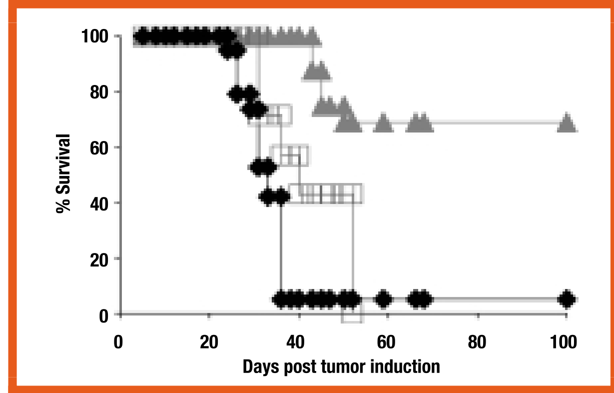

Fig. 2B: Survival of mice with two tumors is prolonged compared to mere contralateral treatment (data not shown). Addition of irradiated tumor cells could not improve survival of contralaterally treated mice (n = 6).

margin of the tumor (peritumoral) or into the opposite flank (contralateral) until treatment was stopped on day 38. Mice with injections into the opposite flank showed reduced tumor growth but finally succumbed to the tumor (Fig. 1). The survival time was not affected when the injection of CpG ODN was combined with irradiated tumor cells. When CpG ODN was injected into the tumor margin, 17 of 20 mice completely rejected the tumor. Coinjection of irradiated tumor cells did not improve peritumoral treatment.

3.2. Peritumoral injections with CpG ODN induce systemic immune responses

To test the development of a systemic anti-tumor immune response in mice bearing two tumors, CpG ODN 1826 was injected into the margin of one tumor only, whereas the other was left untreated.

As a striking finding, the tumor on the non-treated flank also responded to treatment (Fig. 2A). 70 % of the mice survived, completely rejected both tumors and showed long term survival (Fig. 2B). A vaccine consisting of CpG ODN and irradiated tumor cells injected into the contralateral flank of mice with only one tumor showed only little prolongation of survival. Mice which completely rejected the tumor were protected against rechallenge with C26 cells but not with a control tumor demonstrating tumor antigen-specific long term memory.

3.3. Coinjection of CpG ODN improves the therapeutic activity of tumor antigen-pulsed mature dendritic cells in vivo

In a first setting, two injections with CpG ODN 1826 alone, antigen-loaded DC alone or a combination of

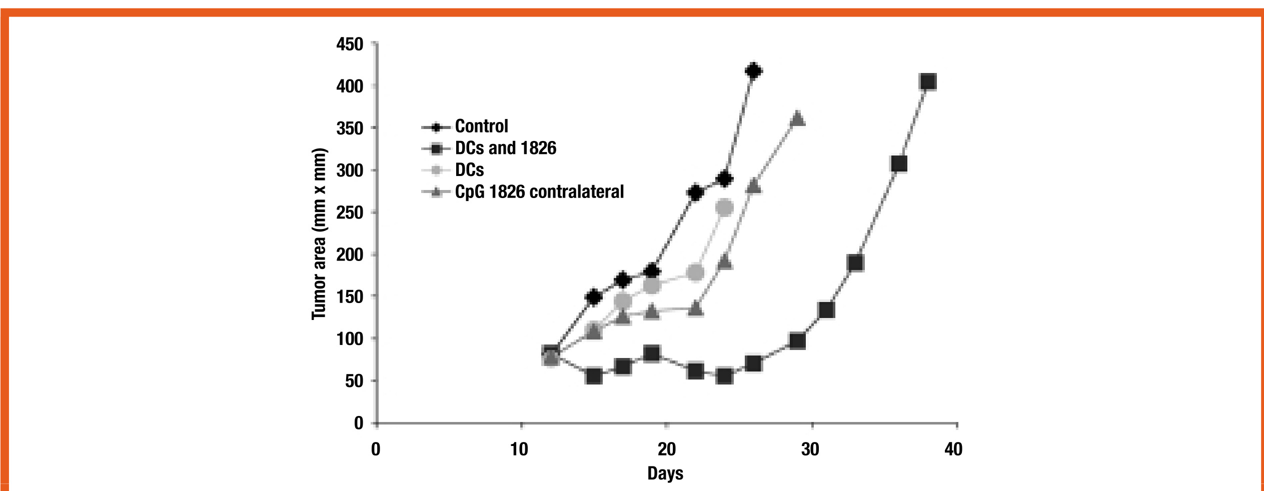

Fig. 3: Two contralateral injections of CpG ODN 1826 alone ▲, DC alone • or CpG combined with antigen-loaded DC ■ were performed. As a control ◆, a group of mice was left untreated. On day 21 the two or three mice that showed the largest reduction in tumor growth were killed for histological examination.

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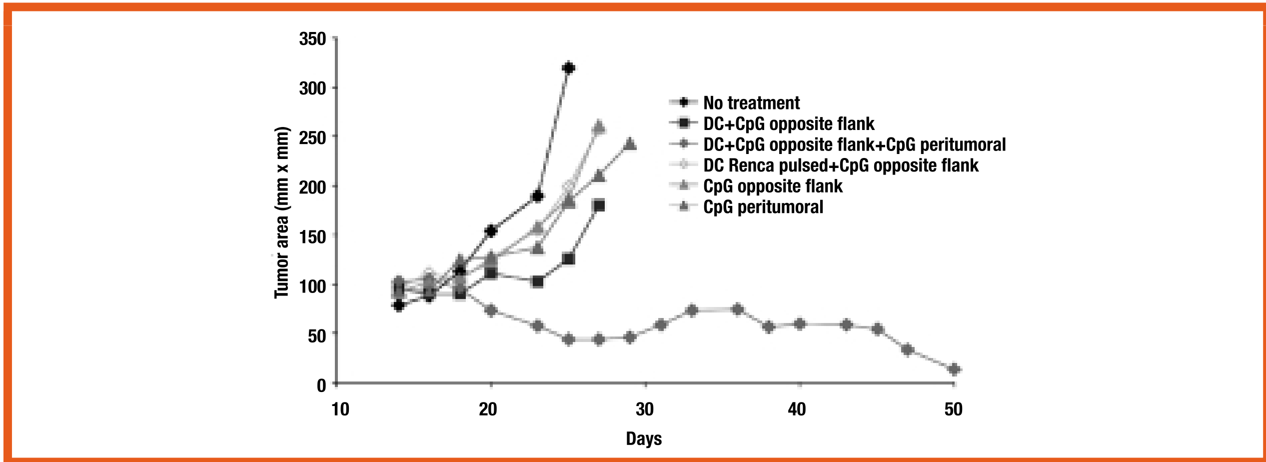

Fig. 4: The anti-tumor activity of DC is tumor antigen specific. Therapy was started when mice were bearing C26 tumors of an average size of 90 mm^2. When DC were pulsed with a control cell line (Renca kidney cancer cells), no difference was observed between CpG ODN 1826 (CpG) injected into the opposite flank of the tumors and a combination of these DC and CpG injected contralaterally. The effect of both therapies was poor. Coinjection of C26-pulsed DC with CpG at distant sites of the tumor allowed a stagnation in tumor growth, whereas the therapeutic effect of peritumoral CpG was limited by the tumor size. Again contralateral coinjection of DC with CpG combined with peritumoral CpG —◆— turned out to be the optimal regimen. This was confirmed in three independent studies. There was no therapeutic effect with activated and C26 pulsed DC alone independent on the site of injection.

both were performed on day 12 and day 17 after C26 tumor challenge into the opposite flank of the tumor. As expected, concerning our previous studies, the effect of contralaterally injected CpG alone was poor, but also DC given alone could not reduce tumor growth. Coinjection of CpG and DC potently improved contralateral treatment leading to a prolonged survival until the first mouse died on day 37 (Fig. 3). The optimal regimen was found to be a coinjection of CpG with antigen-loaded DC at sites distant of the tumor and simultaneous injection of CpG ODN into the tumor margin (Fig. 4). Growth of tumors with a size of up to 220 mm^2 could be controlled during the course of therapy.

3.4. Comparison of chemotherapy and immunotherapy

To compare the efficacy of immunotherapy and chemotherapy of the same systemic toxicity mice were treated with the highest tolerated dose of 5-fluorouracil/leucovorin regarding to major weight loss or underwent the immunotherapeutic protocol. As result the optimal immunotherapy cures 5 of 9 mice, while all of untreated or with low-toxic chemotherapy treated mice succumbed to the tumor (Fig. 5).

4. Conclusions

In an earlier protocol DC were coincubated with irradiated tumor cells and activated by CpG ODN 1826 in vitro. Injection of these DC on day 5 after tumor challenge induced a tumor-specific immune response which cured mice [1]. In this protocol no CpG ODN was present in vivo. In a second protocol weekly injections of CpG ODN without DC into the margin of the tumor lead to rejection of local and distant established tumors.

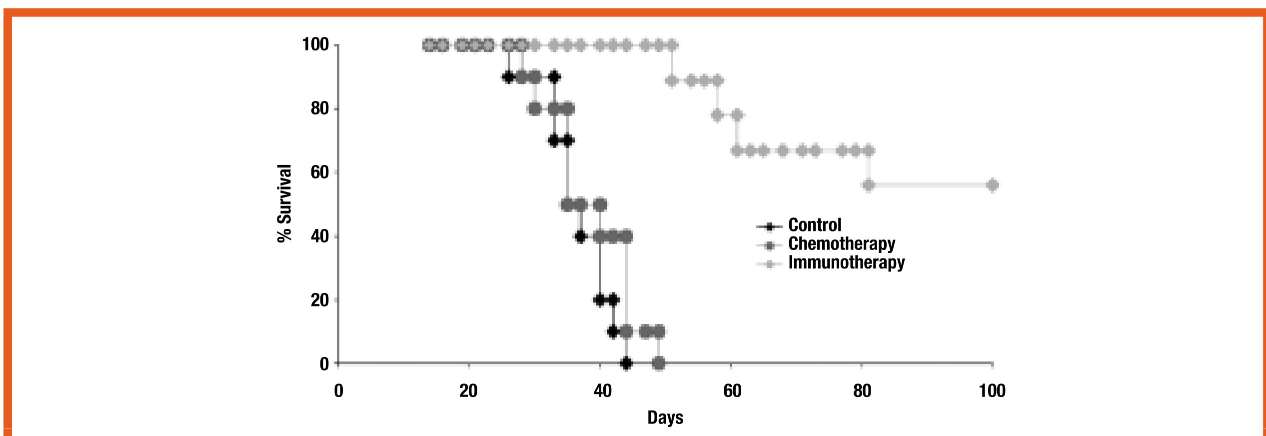

Fig. 5: Therapy was started when mice were bearing C26 tumors of an average size of 65 mm^2. The maximal tolerated dose of 5-fluorouracil/leucovorin (n = 10) (determined by weight loss, data not shown) could not protect mice against death. Immunotherapy consisting of a peritumoral CpG ODN injection and a coinjection of tumor-pulsed DC with CpG ODN 1826 into the opposite flank (n = 9) cured 5 of 9 mice besides no toxicity.

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The generation of an effective systemic anti-tumor response depends on the presence of CpG ODN in the area of vital tumor tissue rather than administration in conjunction with non-proliferating irradiated tumor cells. In a third approach we combined both protocols. CpG ODN were coinjected with antigen-loaded DC in mice with large established tumors. Coinjection of CpG ODN potently improved efficacy of DC. The optimal regimen was found to be coinjection of DC and CpG at sites distant of the tumor and simultaneous injection of CpG ODN into the tumor margin. In conclusion, we established an optimized protocol based on a combination of DC and CpG ODN which allows the control of large established tumors. These results form the basis for clinical studies testing DC and CpG ODN for the treatment of human cancer.

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Recent Progress in the Understanding and Clinical Development of Immunostimulatory Oligonucleotides

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Bacterial DNA is recognized by cells of the immune system based on its CpG content and the presentation of specific CpG motifs. Synthetic oligodeoxynucleotides (ODN) containing these motifs directly stimulate B cells and a specific subset of dendritic cells, the plasmacytoid DCs, through the Toll-Like receptor 9 (TLR 9)and thereby inducing broad spectrum immune defense mechanisms that have therapeutic activity in animal models of infectious and allergic disease and cancer. Coley Pharmaceutical is developing CpG molecules as TLR 9 agonists and antagonists. In Phase I and II human clinical trials our leadproduct CpG 7909 was relatively well tolerated and demonstrated strong adjuvant activity for a hepatitis B vaccine, even in vaccine nonresponders. Additional trials in various cancer indications using CpG 7909 as a monotherapy demonstrated antitumor activity in advanced cancer patients. Furthermore interesting clinical data were observed using combinations of CpG 7909 together with monoclonal antibodies in non-Hodgkin-lymphoma and breast cancer. In combination with tumor vaccines clinical collaborators recently demonstrated anti-tumor activity using CpG 7909 as a strong Th1 immunostimulator together with a tumor antigen in Stage IV melanoma patients.

In the field of asthma and allergy our partner Aventis recently nominated CpG 7279 for clinical development.

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Clusterin Antisense of Mice and Men

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Clusterin, also known as testosterone-repressed message-2 (TRPM-2) or sulfated glycoprotein-2, has been implicated in a variety of physiological and pathological processes; an increasing body of evidence focuses on its role in apoptotic cell death. Based on this property and data earmarking clusterin as a treatment-resistance factor in a number of human malignancies, clusterin has recently received considerable attention as a therapeutically attractive molecular target. Despite the attention, the specific molecular mechanism responsible for clusterin's role in apoptosis remains elusive. Chaperone-like functions have been proposed. We recently addressed this issue in human melanoma and could demonstrate that clusterin up-regulates the expression of the anti-apoptotic Bcl-2 family member Bcl-xL. Notably, overexpression of clusterin up-regulated Bcl-xL, whereas treatment with the advanced chemistry 2'MOE clusterin antisense inhibitor OGX-011 (OncoGenex Technologies Inc., Vancouver, Canada) lowered the expression of this key regulator of programmed cell death. Neither clusterin overexpression by transfection nor clusterin antisense treatment altered the expression of other Bcl-2 family members in the systems tested. Preclinical data support the therapeutic potential of OGX-011 in NSCLC, prostate, breast, renal, ovarian, and bladder cancer as well as melanoma. Additional cancer indications are under investigation. OGX-011 is currently being evaluated in clinical trials in prostate cancer under conditions of hormone ablation and as a chemosensitizer for docetaxel in clusterin expressing solid tumors.

Antisense Therapy in Hematology and Oncology

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There is currently new hope to develop selective anticancer drugs with less cytotoxic side effects than conventional cancer chemotherapy. This optimism is based on the identification of new cancer-associated molecular targets, which would allow the selective targeting of cancer cells while sparing normal cells. Several approaches are available to specifically manipulate gene expression on the DNA or RNA level. Gene therapy uses integration of new genetic material into the genome. This approach can replace defective genes or block the effects of unwanted genes by the introduction of a counteracting gene. Gene expression can also be influenced on the transcriptional level by triple helix forming oligodeoxynucleotides (ODN). In this setting there is no stable integration of genetic material into the genome. An alternative strategy is to use single stranded ODN, so-called AS ODN to modify gene expression on the translational level. This approach is not called 'gene therapy' since the target is mRNA rather than a gene [1].

The number of clinical AS trials represents the growing interest in this technology [2]. In general, systemic AS ODN treatment is well tolerated and side effects are dose-dependent. Dose-limiting toxicities are thrombocytopenia, hypotension, fever and asthenia [3, 4].

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Furthermore, elevation of the liver enzymes aspartate aminotransferase and alanine aminotransferase, as well as complement activation and a prolonged PTT have been reported [5].

In 1998, the first AS drug (fomivirsen, Vitravene®, Ciba Vision) has been approved by the Food and Drug Administration (FDA) for the treatment of cytomegalovirus-induced retinitis in patients with AIDS. Although fomivirsen is administered locally (intravitreal injection), the FDA approval demonstrates the feasibility of AS ODN as therapeutic agents for the treatment of human diseases [6]. A number of AS ODN are currently tested in clinical trials including ODN that target bcl-2, protein-kinase-C alpha and Raf kinase. The clinical studies indicate that AS ODN are well tolerated and may have therapeutic activity. In this overview, we summarize therapeutic concepts, clinical studies, and new promising molecular targets to treat human cancer with AS ODN [7].

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Phase I/II Study of Bcl-2 Antisense Oligonucleotides (Genasense™, G3139) and Dacarbazine in Patients with Malignant Melanoma

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Genasense, an antisense oligonucleotide targeting bcl-2 mRNA, is in Phase III clinical trials for hematologic and solid tumors. We previously reported major biologic activity (down-regulation of Bcl-2 protein in melanoma biopsies) and clinical responses in 12 patients with advanced melanoma after treatment with a 14-day continuous intravenous infusion (CIV) of Genasense in combination with dacarbacine (DTIC) [1]. In the meantime, a total of 33 pts received DTIC combined with Genasense doses ranging from 0.6 up to 12 mg/kg/d. Alternatively to the 14-day CIV, a 5-day CIV schedule as well as 6-day schedule with twice daily sc injections of Genasense were evaluated, both combined with DTIC as bolus iv infusion administered over 1 h. All combination treatment schedules of DTIC and Genasense were well tolerated. Fever, chills, fatigue and transient elevations of liver transaminases were observed as predominant side effects. DLT was reached at a dose of 12 mg/kg/d in form of thrombocytopenia with no clinical sign of bleeding. Mean C_{ss} of Genasense ranged from 1 to 10 µg/mL, linear with delivered dose. Notably, only dose levels above 7 mg/kg/d resulted in relevant increase of IL-6 and IL-12 plasma levels coinciding with fever peaks and thrombocytopenia. Response data from pts with adequate follow-up will be presented. Based on the encouraging results of this Phase I/II trial, the concept of chemosensitizing malignant melanoma with Bcl-2 antisense oligonucleotides is currently being evaluated in a randomized Phase III comparing the combination of Genasense at a dose of 7 mg/kg/d and DTIC vs DTIC alone.

Reference

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A TGF-beta-2 Specific Antisense Oligonucleotide (AP12009) as Continuous Intratumoral Treatment of Recurrent High-grade Glioma Patients

Results of clinical phase I/II studies

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In two phase I/II dose escalation studies patients with high-grade glioma (WHO grade III and IV) have been treated intratumorally with a single course (first study) or a second course (second study) of the tumor necrosis factor (TGF)-beta-2 antisense phosphorothioate oligonucleotide AP 12009 by high-flow microperfusion and demonstrated an excellent safety and tolerability. In a third study, a continuous infusion of up to ten courses of AP 12009 – alternating with NaCl infusion during the intervals – was applied.

AP 12009 was developed to block the mRNA of transforming growth factor beta-2 (TGF-beta-2), which is known to correlate with bad prognosis in high-grade glioma. TGF-beta is the most potent immunosuppressor known.

AP 12009 reversed TGF-beta-2 induced T-cell immunosuppression and reduced glioma cell proliferation and migration in vitro. Toxicology studies in rodents and primates revealed excellent safety and tolerability after both systemic and local application.

The inclusion criteria were identical to those of the preceding study: adult patients with recurrent high-grade glioma and evidence of tumor progression on magnetic resonance imaging (MRI) despite previous therapies (surgery, radio- and/or chemotherapy). The highest tolerated dose in the first study was chosen for one cohort in this study; in the second cohort the duration of the active drug periods was prolonged, using the same daily dose, thus increasing the total dose per course once more. As compared to the first cohort in the first study, the dose was escalated 113fold.

Both AP12009 and NaCl in the intervals were applied by convection enhanced delivery (CED), with the intratumoral implanted catheter connected with an indwelling tube and a subcutaneous port to an external pump, delivering the substances with a continuous flow. During intervals, NaCl was applied at a very low flow rate, to inhibit blocking of the intratumoral catheter tip.

Only in 6 of the total 25 patients possibly drug related adverse events were observed, mostly of grade 1 or 2. There were no changes in the laboratory values. The MTD has not been reached, as evaluated by an independent data and safety monitoring board (DSMB). Also the application system and the CED were tolerated without problems, and very well accepted by both physicians and patients.

Thus far, the 20 patients from the first two studies have been evaluated for efficacy: as per March 21st, 2003, the median overall survival of patients with anaplastic astrocytoma (AA) after recurrence is 77 weeks, and 42.4 weeks for glioblastoma (GBM) patients. The figures for the 13 patients having received temozolomide as chemotherapy before AP12009 are 106.4 weeks for AA, and 46.1 weeks for GBM, respectively. One patient having shown a complete response in all tumor sites is still alive 196.4 weeks after the recurrence (110 weeks after start of AP 12009) – following only one course of AP 12009 therapy.

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Clinical Development II: RNA and Regulatory Aspects

Towards the Development of Therapeutics on the Basis of Small Synthetic Double-stranded RNA Duplexes

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Small chemically synthesized RNA duplexes ("SIRPLEX") have been optimized with reference to inhibitory power in several mammalian cell lines, and stability in murine and human blood serum. Repetitive intravenous injection of chemically unmodified siRNAs without any particular delivery system or conjugate gave rise to distinct reduction of the GFP expression level in several organs of GFP-transgenic mice. Moreover, in xenograft mouse models of human tumors (malignant melanoma, pancreatic carcinoma), a decrease in the tumor growth rate upon application of specific siRNAs was observed. The potential of the siRNA approach for the development of drugs is further demonstrated by means of in vitro models with relevance to different severe diseases, as, e.g., hepatitis C, or acute and chronic myeloid leukemias.

Development of Gene Transfer Medicinal Products

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Gene transfer medicinal products (GT-MPs) are medicinal products (i) which are either used with the aim of genetically modifying human somatic cells in vivo, or (ii) which consist of or contain ex vivo genetically modified autologous, allogeneic or xenogeneic cells. Genetic modification of cells, either ex vivo or in vivo, involves the use of replication incompetent viral vectors, non-viral vectors or "naked" nucleic acid. In some cases, replication competent microbes such as replicating adenovirus are being used. Viral vectors commonly used include retroviral, adenoviral, adeno-associated virus (AAV) and pox virus vectors the safety and efficacy profile of which are different. Therefore, each vector is commonly used in specific settings. The modified cells used in vivo include autologous, allogeneic or xenogeneic cells.

No marketing authorisation of a GT-MP has been granted and clinical trials, so far mainly carried out in Europe and North America, have been concentrated on phase I/II with a large emphasis on analysing safety and obtaining first evidence for possible efficacy of GT-MPs. Disease targets include monogeneic diseases, cancer, infectious and cardio-vascular disease, examples of which show that each disease may need the development of a particular approach and the use of a particular vector, expression construct or modified cell. With an increasing understanding of the underlying molecular biology, first evidence of possible efficacy has been obtained in a number of clinical trials including those targeting ischemia, head-and-neck cancer, hemophilia and monogeneic disease. However, proof of efficacy has yet to be obtained in phase III studies. With the improvement of gene transfer efficiency, serious adverse reactions are also being observed and will have to be met by improving the vectors and strategies applied.

In the European Union, marketing authorisation procedure via the European Agency for Evaluation of Medicinal Products (EMEA; Council Regulation (EEC) No.

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2309/93). Experts from national authorities like the Paul-Ehrlich-Institut (http://www.pei.de/themen/themlink.htm) are involved in the review of applications and scientific advice given to the developing pharmaceutical industry. A European 'Note for Guidance' on the preclinical and clinical aspects of GT-MPs is available. For the evaluation and review of clinical protocols, a parallel procedure involving the approval by a competent authority and the positive appraisal of the local ethics committee will be established (Directive 2001/20/EC). General issues and matters of importance to the field of gene therapy will be discussed with experts under the auspices of the Gene Therapy Expert Group of the CPMP/EMEA.

Conclusions and Remaining Problems

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The conference will have been concentrating on three applications of oligonucleotides, two of which relate to the antisense technology, the other to the immune stimulation by CpG-containing oligonucleotides. Data will have been presented on the efficacy of the three types of oligonucleotides as therapeutics with the emphasis on clinical settings, providing a comparison of the advantages and disadvantages of the two antisense approaches, one with the first and second generation of oligodeoxynucleotides, the other with siRNAs. The CpG-containing oligomers can not directly be compared to the other two as they don't act on the mRNA level.

All applications of oligonucleotides as therapeutics share common problems such as identification of accessible sites on a target RNA, delivery independent of exogenous or endogenous application, and specific targeting of certain cell types. Further desirable features are oral delivery and the design of oligonucleotides to penetrate the blood-brain barrier. Further stabilisation of the oligonucleotides could still become an issue at a later stage. All of these issues require further research.

Three types of oligonucleotides have not been considered here, ribozymes, fully 2'-modified oligonucleotides and aptamers. Their development should not be lost sight of.

In conclusion, various types of oligonucleotides rend themselves at least potentially as therapeutics. Further work is needed to bring several of them to the status of approved drugs.

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