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Killing cancer by targeting genes that cancer cells have lost: Allele-specific inhibition, a novel approach to the treatment of genetic disorders

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Abstract. Oligonucleotide-based drugs are now rapidly establishing themselves as an important tool in both research and treatment of genetic disorders. In the past many problems were encountered in using antisense oligonucleotides. Our expanding knowledge and new oligonucleotide chemistries are giving us the chance to treat serious genetic disorders such as cancer in novel, elegant and effective ways not previously possible. In addition, recent knowledge about RNA interference may add to these new approaches for disease treatment with oligonucleotide-based drugs. In this review we discuss one such novel therapeutic strategy against cancer called allele-specific inhibition (ASI). ASI is an approach where cancer cells are attacked at one of the few widely occurring and consitently weak points: the loss of large segments of DNA. Oligonucleotide-based drugs may provide the required selectivity for this therapeutic approach.

Key words. Antisense oligonucleotides; RNA interference; SNPs.

Introduction

The development of therapeutic agents which target tumor cells with high specificity relative to normal cells of the body has been a fundamental challenge in cancer research. Because tumor cells retain most of the genetic characteristics of normal somatic cells, this challenge is a difficult one to address. Some of the most successful cancer treatments to date capitalize on the genetic differences between normal and tumor cells. These genetic windows of opportunity are neither easy to discover nor straightforward to exploit. A novel therapeutic strategy against cancer, termed allele-specific inhibition (ASI), has been proposed which systematically exploits a class of genetic differences between tumor and normal cells which are perhaps the most widely occurring source of genetic variation between tumor and normal cells. ASI involves attacking cancer cells through vulnerability created by the loss of large segments of chromosomal material, the so-called loss of heterozygosity (LOH), a consequence of mitotic non-dysjunction during the process of tumorigenesis. The vulnerabilities created via LOH are one of the few (if not only) widely occurring, absolute, and consistent Achilles' heels exhibited by tumor cells. The rationale for ASI therapy is based on the fact that a site in the genome which is heterozygous in normal somatic cells can become homozygous in tumor cells as a consequence of LOH. If such a polymorphic site occurs in a gene required for cell viability, then an ASI for the allele remaining in the tumor cell will result in selective tumor kill, while normal somatic cells will remain viable because the allele not targeted by the ASI will provide the necessary biological function in the normal cell. The most attractive targets for ASI therapy are nucleic acid targets, specifically messenger RNAs (mRNAs) which encode functions essential for cell viability. Currently, the agents most able to achieve the selectivity and specificity required to implement this approach are nucleic acid-based therapeutic agents, including antisense oligonucleotides and double-stranded RNA (dsRNA) molecules capable of causing RNA interference. We may be on the

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verge of a renaissance in oligonucleotide-based drugs, which may make the use of ASI-based therapies a practical reality. Fifteen years after the initial hype and years of subsequent sceptism, several first-generation antisense oligonucleotide drugs are now registered or advancing in clinical trials [1]. The recent development of RNA interference in the last several years may provide an extremely promising approach to the implementation of ASI therapy. In this review we discuss current data on ASI therapy with the goal of evaluating the most promising paths towards achieving this therapeutic approach in the near future.

The battle against cancer

For several decades now, the effort to treat cancer has been described in terms of 'the war on cancer'. A key issue in cancer treatment, by analogy to action on a real battlefield, is that 'friendly fire' should be avoided as much as possible. Most cancer therapies are unable to clearly distinguish friend from foe in this context, and the consequence of many forms of cancer therapy is that the collateral damage to normal cells is often the limitation which prevents treatment from reaching a successful conclusion. The reason for this is that although the goal of cancer therapy is to target drugs against a protein specifically expressed in the tumor cells, in most cases the tumor-specific protein is not only expressed in the tumor cells but also in normal cells. Specificity is achieved because of the high level of expression of the target gene product in the cancer cell or because of biochemical requirements of the tumor cell. Amplified genes or genes with specific point mutations or chromosomal translocations are thus potential tumor-specific targets.

For proteins highly expressed in a tumor cell, antibodies against tumor specific targets can be developed. Herceptin, directed at the Her2-Neu protein, and Iressa, an inhibitor of the epidermal growth factor receptor, which are overexpressed in some forms of cancer (e.g. breast), are used in treatment of metastatic cancer. These drugs, however, are directed against targets which are not really tumor specific. These drugs have some tumor-selective effects because of the high level of expression of the target in the tumor cell. However, mutations in genes involved in the signaling pathway regulated by these receptors may result in drug resistance.

Development of drugs against a target that is specific for tumors because it is not encoded by the genome of the normal cell is in principle a more effective approach to the specificity problem. Thus far, only a few drugs directed at these tumor-specific targets have been developed. Glivec, ST 571, specific for the *BCR-ABL* tyrosine kinase was one of the first anticancer tyrosine-kinase inhibitors based on rational drug design. The *BCR-ABL* fusion gene encodes the BCR-ABL tyrosine kinase, which is the product of the Philadelphia chromosome translocation specific for chronic myelogenous leukemia. A major drawback of the drugs described above is that they are specific for only a subset of tumors.

A novel and theoretically even better way to attack cancer cells specifically is not to attack cancer cells based on their genetic gains compared with normal cells, but rather through vulnerabilities created by genetic losses. Many types of cancer cells lose genetic sequences irreversibly during tumorigenesis, which may be exploited as a vital weak point in their defense. The loss of large chromosomal regions, or even whole chromosomes, is an early event in the clonal evolution of cancers. Regions involved in LOH are thought to contain tumor suppressor genes, and LOH can involve >20% of the total genome in certain cancers [2]. Hence, the genetic difference between normal cells and cancer cells extends beyond the loss of a tumor suppressor gene. Many genes will be reduced to hemizygosity in cancer cells due to LOH, and some of these genes will be essential for cell survival. Thus, LOH represents an irreversible difference between normal and tumor cells, and this forms the basis for ASI. The basic principle of ASI is given in fig. 1.

Requirements for ASI

ASI is based on the the following rationale: (i) tumor cells carry large regions of LOH; (ii) LOH regions contain genes essential for cell survival; (iii) these genes show genetic variation in their mRNA-coding sequences; (iv) both alleles are expressed in normal tissue; (v) inhibition of the allele retained in the tumor is cytotoxic and can be achieved by ASI.

During the last 5 years data have become available that support the underlying assumptions for ASI, and the first in vivo experiments showing proof of principle for ASI have been completed. In the following sections we will review the data that provide support for ASI as a viable approach for cancer therapy.

In order to make ASI possible, the following questions have to be answered.

1) Is there sufficient genetic variation to allow ASI to become a viable approach?
2) Are sufficient essential genes located in regions of LOH?
3) Can the remaining alleles of essential genes in tumor cells be inhibited specifically, resulting in specific tumor cell kill, and what type of drug is most suited for this work?

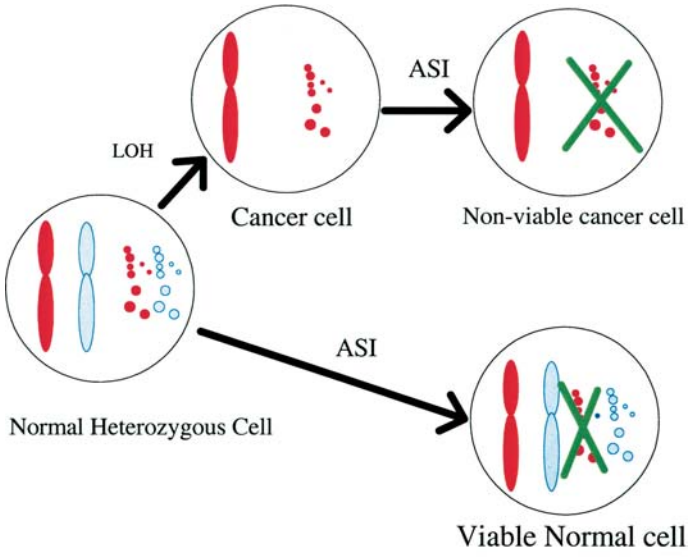

Figure 1. ASI of a target on chromosome 17 will be cytotoxic for the hemizygous cancer cell, whereas the normal heterozygous cell will be unaffected. LOH can, of course, also occur for only a part of a chromosome.

Genetic variation in genes encoding essential function

Determining the frequency of polymorphic sites in the genome which could become the targets of ASI was a critical first step in exploring the feasibility of ASI. Initially, it was estimated that 1 in 300 nucleotides in the human genome is polymorphic, based on studies of disease-related genes [3]. Sequencing large regions of DNA around the APO E gene yielded polymorphism frequencies of about 1 : 1100 [4]. This suggested that either polymorphisms are not equally distributed over the genome, or that many occur at low frequency in non-disease-related genes. To address this issue, we selected 22 highly conserved genes for polymorphism screening, all of which have homologs in *Saccharomyces cerevisiae* and therefore are likely to encode functions necessary for cell viability (table 1). Of these genes, 19 of 22 are in the apparent minimal gene set required for cellular life [5, 6]. This is a collection of 250–300 genes derived from a comparison of the genomes of the distantly related prokaryotes *Mycoplasma genitalium* (which has 468 predicted genes, the smallest known gene complement in a cellular life form) and *Hemophilus influenza* (which has 1703 predicted genes). The three other genes selected are eukaryotic initiation factor 5A, CTP synthetase and the 30-kDa TATA-associated factor (TAFII30). The former two have clear functional counterparts in prokaryotic genomes. The extent of amino acid identity between human and yeast homologs is at least 27% and ranges up to 82%, whereas the degree of cross-species amino acid similarity ranges from 44 to 88%. In view of the interspecies conservation of these genes, it is likely that they have been under continuous selective pressure for millions of years. Therefore, it might be expected that they exhibit lower divergence, or a different distribution of polymorphic changes, than other, less conserved genes. We have analyzed the mRNA sequences of these 22 essential genes (>51 kb mRNA) for polymorphisms in 36 unrelated individuals of different ethnic origin. Sixty-five polymorphisms were detected, an average of 3 per gene. Sixty were single-nucleotide polymorphisms, five insertions and one complex alteration was detected. 1 : 738 nt is polymorphic. These results indicate that there is a significant and sufficient frequency of polymorphisms in sequences of essential genes in the human population [7]. The results are summarized below in table 1.

Are essential genes affected by LOH in tumors?

Human tumors can lose up to 20% of their genome [2]. Allelotype data show that very large regions of the genome can be lost [8]. A large set of breast, ovarian and non-small-cell lung cancers was tested by Basilion et al. [9]. LOH at the RPA70 locus which is also in proximity of TP53 (band 17p13.3), was determined for 189 paired normal and cancer tissues from patients selected for constitutional RPA70 heterozygosity. These studies showed LOH for RPA70 in 44% of colon cancer, 58% of ovarian cancer, 19.5% of breast cancer and 27% of non-small-cell lung carcinoma. We have used the polymorphisms identified in the large subunit of RNA polymerase II (POLR2A) to test whether gliomas show LOH for POLR2A as well. Gliomas frequently show LOH of TP53 (band 17p13.1), which is in close proximity to POLR2A

Table 1. Polymorphisms in highly conserved genes.

Gene	Position	Polymorphism	Heterozygosity	Mutation	Sequence flanking polymorphism
AARS	1013	T>C	56	silent	TGGCTGACCA **T** GCTCGGACCA
CARS	1739	C>T	44	silent	ACATCCTGCC **C** GAGCTTGGGG
EPRS	2520	C>A	6	P821H	AATTCTGAAC **C** TGCTGGTTTA
	2944	G>A	3	silent	TCATCACAAA **G** TCAGAAATGA
	2963	C>T	3	H969Y	GATTGAATAC **C** ATGACATAAG
	2969	A>G	22	I971V	ATACCATGAC **A** TAAGTGGCTG
	3247	A>G	44	silent	AATGGGTACA **A** TCACACAGAG
	4459	G>A	50	3′-UTR	GATACAGACC **G** TTTTATGATT
	4506	G>A	50	3′-UTR	AAGTCACACA **G** GACAATTATT
IARS	2905	T>C	8	silent	AAAACAAGTA **T** GGCATTCGGC
KARS	89	A>G	19	silent	AGCTGAAGAG **A** CGCCTGAAAG
	1789	G>C	6	S595T	ACAGTTGGCA **G** TTCTGTCTAG
QARS	404	C>T	11	silent	TTAACAGGCA **C** CGGCCCCAGC
TARS	1608	G>A	14	silent	CTACTCGCCC **G** GAAAAATTCC
	1755	G>A	31	silent	TTAAAGATGC **G** ATTGGGCGGT
	2395	T>C	25	3′-UTR	TGGCAAAGTC **T** GAAATAGGTC
VARS	1100	G>C	3	G294A	GATGTCAGTG **G** CCCCATGCCC
	1287	C>G	3	silent	GCCATGCACT **C** ACCAACGCCA
	2096	C>T	3	S626F	CAATGTGCCT **C** CGCCTTTCCT
	2847	C>T	25	silent	ATCCCCTGGA **C** GTCATCTATG
	3385	G>A	3	V1056I	TTGCCTGGAC **G** TTGGCCTGCG
RPA1	81	G>A	25	silent	TGGTCGGCCA **G** CTGAGCGAGG
	1120	A>G	25	A351T	CTTGATGGAC **A** CATCCGGGAA
	1125	C>T	25	silent	TGGACACATC **C** GGGAAGGTGG
	1674	T>C	31	silent	TCCAGGAGTC **T** GCTGAAGCTA
	2046	T insertion	3	3′-UTR	GACTAAGCAA (**T**2) CCTCCCTCGT
	2050	T>C	42	3′-UTR	AAGCAATTCC **T** CCCTCGTGCG
	2296	C insertion	33	3′-UTR	GTGGTGACCA (**C**8) ATCCCCGCTC
	2341	G>A	3	3′-UTR	TCAGCGGGGC **G** AGCTGAGAAG
RPA2	252	T>G	14	L59V	TTCTGCCACT **T** TGGTTGATGA
	313	T>A	31	I79N	ATTGTGGGGA **T** CATCAGACAT
TAFII30	554	G>A	22	silent	TGAAGGGCAC **G** GCCTCCGGCA
RPS6*					
RPS14	183	G>A	44	silent	TTTCTGGCAA **G** GAAACCATCT
RPL7A	421	G>A	8	silent	ACGTCCCAAC **G** AAGAGACCAC
RRM1	1037	C>A	31	silent	CAACACAGCT **C** GATATGTGGA
	2410	G>A	39	silent	ATTTAAGGAC **G** AGACCAGCAG
	2419	A>G	19	silent	CAAGACCAGC **A** GCTAATCCAA
	2717	T>A	19	3′-UTR	GTTAATGATG **T** TAATGATTTT
	2724	T insertion	3	3′-UTR	ATGATAATGA (**T**9) AAACTCATAT
RRM2	524	C>G	3	Silent	TTGACCTCTC **C** AAGGACATTC
	1399	T>A	3	3′-UTR	TGGCTGATTT **T** TTTTTTCCAT
	1636	C>T	3	3′-UTR	CTTTAGTGAG **C** TTAGCACAGC
	2259	T>C	3	3′-UTR	TAAGGTAGTA **T** TGTAAAATTT
TYMS	1140	C>T	53	3′-UTR	CAAAGGAGCT **C** GAAGGATATT
	1210	A>G	42	3′-UTR	TCTAAAAGAA **A** AAGGAACTAG
	1571	A>T	53	3′-UTR	ATGAACTTTA **A** AGTTATAGTT
DHFR	721	T>A	11	3′-UTR	CTAAGCAACT **T** GTTTTTATTC
	829	C>T	3	3′-UTR	GCACCTGCTA **C** AGTGAGCTGC
CTPS	576	A>G	6	silent	GTCAGTTCCA **A** TTCAAGGTCA
	2093	C>T	3	3′-UTR	CAGAACATCG **C** GATGGGAACC
	2135	G>A	8	3′-UTR	TGTCCCCATC **G** GTCACCTTGT
POLR2A	857	G>A	22	silent	GCGAGGGTGG **G** GAGGAGATGG
	1260	C>T	6	R292C	TCAGCTGCGG **C** GAATGAGCA
	1346	T>C	39	silent	TGGTGGACAA **T** GAGCTGCCTG
	1544	C>T	3	silent	CCATTGCTGC **C** AACATGACCT
	1847	C>T	31	silent	TGAATCTTAG **C** GTGACAACTC
	2678	C>T	11	silent	CTGAATACAA **C** AACTTCAAGT
	3059	C>T	31	silent	AGCTGCGCTA **C** GGCGAAGACG
	3827	C>T	22	silent	TGGGCCAGTC **C** GCTCGAGATG
	6466	T>C	50	3′-UTR	CTGATGCAGA **T** TCTTGTCTTG
	6557	T>C	6	3′-UTR	TGTCCCCAAA **T** TGAAGATCCT
POLR2C	215	C:>T	28	silent	ATTCCTCAGT **C** CTTCATGATG
	3′-UTR	complex	11	3′-UTR	complex repeat differences
eIF5A	623	A>G	36	3′-UTR	GGCTCCCAGG **A** TGGCGGTGGT
	1012	C>T	47	3′-UTR	CCCTGTTGCC **C** ATAGCCCTTT

'Heterozygosity' is given as percentage for the total of 36 samples. The ethnic groups are too small to give reliable data on SNP distributions in subsets. Polymorphic nucleotides are given in boldface. 3′-UTR, 3′-untranslated region. *No polymorphisms were found in the coding region and 3′-UTR of the RPS6 gene.

(band 17p13.1) [10]. Seven of the 10 tumors tested were informative, and all show LOH for POLR2A. These studies show that regions of LOH in multiple tumor types often extend over large regions and can affect multiple (essential) genes. Since TP53 is one of the the most mutated tumor suppressor genes identified thus far, POLR2A and RPA70 are potential targets for ASI. Due to their close proximity to TP53 they represent targets for a large group of tumors.

Oligonucleotides as candidate drugs for ASI

The majority of the high-frequency sequence variants that have to be targeted are neutral substitutions, i.e. they do not alter the amino acid sequence of the gene product. This makes it difficult to find ASIs that act on protein polymorphisms that occur frequently enough within the human population to be of any therapeutic use. Therefore, drugs that can specifically recognize nucleotide sequences are best suited for ASI. An important requirement is that the drug used for ASI must be able to discriminate between one single nucleotide polymorphism (SNP). Oligonucleotide-based drugs may be able to provide for these requirements, as discussed below.

Oligonucleotide-based drugs, especially antisense oligonucleotides, provide the potential for selective inhibition of gene expression. Oligonucleotide-based drugs can be used to specifically inhibit or modify gene expression in several ways. First, the 'classical antisense' mode of action of oligonucleotides, where single-stranded synthetic short oligonucleotides (10–30 nucleotides in length) bind directly to the targeted mRNA and thereby affect its translation. Depending on the chemistry of the oligonuclotide used, this is caused either by steric hindrance of the transcription complex or by initiating degradation of the mRNA-oligonucleotide heteroduplex as a result of RNaseH recruitment. Second, short double-stranded (and single-stranded) RNA oligonucleotides can trigger a conserved biological response in cells, efficiently silencing the targeted genes, involving a complex mechanism involving multiple enzymes [11] which is now generally referred to as RNA interference. Third, oligonucleotide-based drugs may bind directly to proteins or noncoding ribonucleotides (both aspecific and specific) acting as small molecular inhibitors. Examples of this nontypical antisense mode of action are the antitelomerase oligonucleotides [12] and the so-called decoy oligonucleotides, which prevent functioning of DNA-binding regulatory proteins [13]. However, the direct binding of antisense oligonucleotides to proteins is generally regarded as the cause of many of the described side effects of treatment with oligonucleotides [14].

Oligonucleotide-based drugs capture the imagination with their promise of rational drug design and theoretical specificity. However, specific antisense oligodeoxynucleotides (ODNs) are far more difficult to produce than was originally anticipated. Initially, stability was one of the problems encountered when using antisense technology. Short sequences of normal DNA are degraded rapidly by cellular nucleases. Chemical modifications have been introduced to enhance stability of the ODNs. Phosphorothioate (PS) ODNs are the most commonly used chemically modified ODNs. They are relatively stable, and when bound to mRNA, the heteroduplex is a substrate for RNase H. However, these PS ODNs have shown a tendency to mediate non-sequence-specific effects due to their polyanionic nature, causing interactions with proteins. This was already observed in the early nineties when antisense ODNs were used as inhibitors of human immunodeficiency virus (HIV)-1 (see overviews [15, 16]). A wide variety of unexpected non-antisense effects have come to light. These side effects make it hard to produce drugs that act primarily through true antisense mechanisms (i.e. sequence specific) and complicate the use of antisense compounds as research reagents. Despite these problems PS ODNs have proved to be very valuable and have shown to be useful compounds in experiments with proper controls. Several oligonucleotides are already in phase II and III clinical trials [1]. These include an inhibitor of H-ras [17], an inhibitor of c-*raf*-kinase [18], an inhibitor of protein kinase C (PKC)-α [19] and intercellular adhesion molecule (ICAM) [20]. Vitravene, directed against CMV-induced retinitis, is the first antisense drug that is registered, and it is expected that at least two more anticancer antisense ODNs (against Bcl-2 and PKC-α) will be registered in the next 2 years.

Although PS-ODN chemistry is far from ideal, low cost and ease of access still makes it the first choice for many researchers interested in antisense ODNs. Antisense ODNs that are used for ASI must be highly specific for one-base mismatches, since SNPs are targeted in ASI. Design of a sequence-specific ODN is usually a case of trial and error. In fact, this holds for the design of all ODNs. The targeted sequence not only has to be unique in the genome, but it also has to be accessible for the ODN. Despite much effort, no good algorithms are available for predicting ODN-accessible sites. Elegant studies by Shen et al. [21] show that mRNA folding and thus accessibility of certain regions for ODNs can be influenced by SNPs. Thus, designing an antisense ODN specific for a SNP depends on both the SNP to be targeted and the sequence variants in the surrounding region, since both can give sequence induced conformational changes. A wealth of literature is available describing attempts to downregulate mRNA sequences using sequence-specific ODNs and several cases of single-base mismatch discrimination have been reported. One of the most-studied cancer-related targets against which single-nucleotide-sensitive ODNs were developed is the *Ha-ras* gene. Codon 12 mu-

tations (G-T) are among the most frequent cancer-related alterations in the *Ha-ras* gene, and several reports describe the targeting of this variant [22–25]. These studies showed that phosphorothioate backbone ODNs could discriminate between the two variants, but that this selectivity was critically dependent upon oligonucleotide length and concentration. Further in vivo studies indicated that non-sequence-dependent effects strongly contribute to the antitumor effects of the anti-*Ha-ras* PS ODNs [26].

Can SNPs be targeted using oligonucleotide drugs?

Thus far three published studies have shown that targets for ASI can efficiently be targeted in an allele-specific way using PS ODNs. Basilion et al. [9] developed ODNs specific for two variants of *RPA70* and ten Asbroek [27] and Fluiter [28] showed that *POLR2A* is a suitable target for ASI in vitro and in vivo. P*OLR2A* is one of the target genes for which allele-specific ODNs were designed, which discriminate between the two alleles differing only in a SNP. It was demonstrated that these ODNs can inhibit the growth of human tumor xenografts in nude mice [28]. The ODNs were administered continuously through osmotic minipumps. As can be seen in fig. 2, the growth of the tumors was only affected by the respective matched ODNs. MiaPaca II (pancreas) tumors, which are homozygous for a T in the target region of *POLR2A*, were only inhibited in their growth rate by the matched ODN (L5Tas17), and not by the single-base mismatched L5Cas16. Similarly, 15PC3 (prostate) tumors, homozygous for a C in the target region, are only affected by the matched L5Cas16 and not by the single-base mismatched L5Tas17. Therefore, the antisense ODNs can discriminate between the two alleles of a SNP in *POLR2A*, and cause genotype-specific reduction of tumor growth in vivo. In addition the mouse *polr2a* sequence differs in one base from the human sequence in the targeted region of the mRNA. Thus, depending of the human genotype, one or two mismatches are present. We observed no effect of the antisense oligonucleotides on the mice. These results indicate that ASI therapy is a potentially viable approach to develop a tumor-cell specific anticancer therapy, and that *POLR2A* is a good target gene for antisense ODN-mediated inhibition of tumor growth. As with *Ha-ras* ODNs, the selectivity for one nucleotide is crucially dependent on the concentration of the ODNs. The allele specificity of the ODNs was reduced when the daily dose of ODNs was increased to 10 mg/kg. At this higher dosage, both L5Tas17 and L5Cas16 could inhibit tumor growth similarly, irrespective of the tumor genotype.

The most important problem for ASI remains the effective discrimination of the two alleles of a SNP. For each target SNP this will be a process of trial and error, since both the antisense ODN and the target mRNA will influence this process. Shen et al. [21] showed that SNPs can

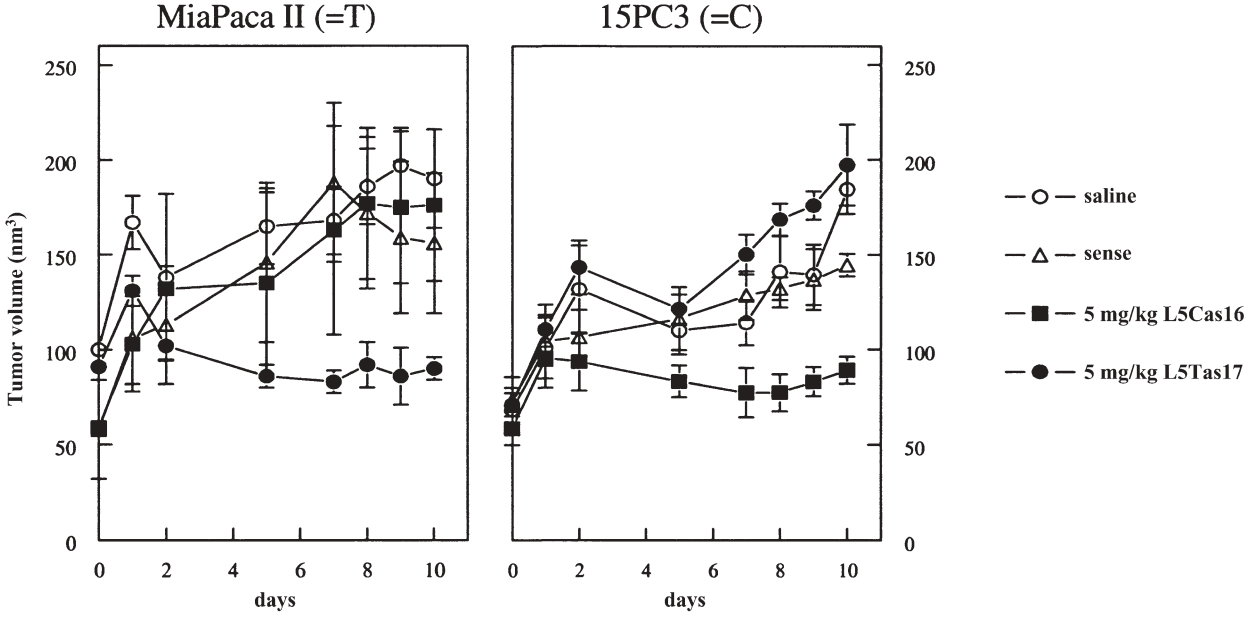

Figure 2. The effect of POLR2A antisense ODNs; L5Tas17 and L5Cas16 on the growth of human tumor xenografts in NMRI nu/nu mice. NMRI nu/nu mice were injected subcutaneously in the flank with (*A*) 10^6 Miapaca II cells (homozygous T for the targeted SNP) or (*B*) 10^6 15PC3 cells (homozygous C for the targeted SNP) from culture in 300 µl of Matrigel. After 1 week of tumor growth the mice received an Osmotic minipump (Alzet model 1002), which was implanted dorsally. The Osmotic minipumps were filled with L5Tas17 (filled circles) and L5Cas16 (filled squares) antisense ODN (5 mg/kg body weight) and sense (5 mg/kg/day) (open triangles) and saline (open circles) controls ($n = 5$ for each group). The tumor growth was monitored following the implantation of the osmotic pump. The results, expressed as tumor volume, are given as means ± S.E.M.

cause marked changes in the secondary structure of an mRNA which may lead to altered accessibility of the mRNA for antisense ODNs and therefore affect the allele specificity of an ODN. For the *POLR2A* SNP target it was found that shortening the ODN, as well as shifting the SNP even a single position within the ODN, could yield major differences in allele specificity and efficacy of the ODN. The number of antisense ODNs that can be used for ASI is limited, since the target is limited to a specific region containing the SNP. This limits the amount of ODNs that must be tested empirically, but also severely limits the freedom of ODN design. We therefore envisage that ASI might not be feasible for every potential target SNP.

Advanced oligonucleotide chemistries

The initial studies of ASI were performed using simple PS antisense ODNs. PS-modified DNA has a relatively low affinity for matching sequences as compared with nonmodified DNA or novel ODN chemistries. These advanced chemistries might further increase the allele specificity and efficacy of ODNs. In addition, their use may limit some of the toxicity issue and side effects associated with the classical PS ODNs. By carefull choices of chemistry and combination of several nucleotide analogs within one oligonucleotide (mixmers), it might be possible to increase the selectivity for single-nucletide mismatches. A number of new oligonucleotide analogs have become available. At this moment, antisense ODNs with different types of chemistry such as peptide nucleic acids, *N*3′,*P*5′-phosphoroamidates, morpholino phosphoroamidates and 2′-alkoxy modifications are under investigation by multiple groups of researchers around the world (for reviews see [29–31]).

The best-known class of new analogs are those with 2′-alkoxy sugar modifications. These 2′ sugar-modified oligonucleotides show high binding affinity to target mRNA, nuclease resistance and higher lipophilicity compared with the classic PS ODNs. Monia et al. [24] reported almost 10 years ago the first evaluation of a series of 2′-modified oligonucleotides containing 2′-deoxy gaps directed against the codon 12 mutated *Ha-ras* sequence [25, 32]. The 2′-alkoxy modifications in general increased the stability against nucleases and improved the target specificity but also introduced a property that limited their usefulness. Most important, these 2′-alkoxy modifications severely inhibit the capacity of these oligonucleotides to recruit RNase H activity, thereby limiting their efficacy to inhibit gene expression. However, the inclusion of a 2′-deoxy gap could restore the RNase H recruitment, and it was concluded that such a gap was quite essential for effective antisense activity. The increase in affinity introduced by the 2′-modified nucleotides in a gapmer ODN greatly enhanced efficacy.

Other classes of modified ODNs include the *N*3′,*P*5′-phosphoroamidate oligonucleotides containing a 3′-amino instead of a 3′ hydroxyl nucleoside [33]. More extensively modified are the morpholino oligomers [34] and peptide nucleic acid oligonucleotides [35] where the natural backbone of ribose is replaced by other structures such as polyamide chains. Like the 2′-alkoxy-modified oligonucleotides, they are all unable to recruit RNase H activity but are able to inhibit gene expression by interfering with the translation machinery when bound to their target mRNA. Unlike the 2′-alkoxy-modified nucleotide analogs which are easily synthesized into mixmer and gapmer ODNs, the more extensively modified morpholinos and PNA ODNs are more difficult to incorporate into gapmer or mixmer structures. This might severely limit their use as allele-specific drugs.

Locked nucleic acids (LNAs) are a novel class of DNA analogs that provide major improvements in a number of key properties. LNAs combine by far the highest affinity ever reported for a DNA analog towards complementary DNA and RNA with a good ability to discriminate between matched and mismatched target sequences [36]. LNAs contain a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon. By virtue of this bicyclic structure, the furanose ring of the LNA monomers is locked in a 3′-endo conformation, thus structurally mimicking the standard RNA monomers. LNA induces large increases in thermal stability (melting temperature, T_m) of duplexes towards complementary RNA (for reviews see [29, 37, 38]).

Recent in vivo studies by Wahlestedt et al. [39] have shown that DNA/LNA copolymers can be used as stable, nontoxic and potent antisense ODNs that are able to recruit RNAseH provided a stretch of DNA (with no 2′-sugar modifications) is present in the ODN. Importantly, this study very clearly showed that LNAs containing ODNs were in vivo more potent in knocking down the rat delta opioid receptor than the classic full DNA ODNs. Kurreck et al. [40] performed a systematic analysis where they compared the stability and ability to activate RNase H and the T_m of LNA/DNA chimeras as compared with classic DNA phosphorothioates and 2′-*O*-methyl gapmers. These studies showed that introduction of LNA into a DNA ODN could greatly enhance T_m and resulted in greater stability in human serum as compared with isosequential phosphorothioates. Full LNA PO ODNs, which have a very high affinity for their complementary mRNA but cannot recruit RNase H, might inhibit protein translation directly without the aid of RNase H recruitment.

Preliminary data show that in vitro full LNA ODNs are very potent in inhibiting one of the targets for ASI, POLR2A protein levels, but at the expense of high sequence specificity in vitro. ODNs with a single mismatched nucleotide or a four nucleotide mismatch were

as effective in inhibiting the POLR2A protein levels in vitro. Surprisingly, when these LNA PO ODNs were administered in vivo, we did observe sequence-specific and dose-dependent inhibition of tumor growth. At a dose of 1 mg/kg/day we observed that 15PC3 tumor xenografts were inhibited in their growth with the matching LNA PO ODN. The other two LNA PO ODNs, with the single- or four-base mismatch, showed less efficacy in tumor growth inhibition, indicating that the observed effects are sequence dependent and 'true antisense' effects. Increasing the dosage led to increased tumor growth inhibitory effects, but at the expense of sequence specificity [40a]. The extreme increase in affinity for the target mRNA of a fully LNA-modified ODN may be too much to create a selective ODN. However, careful placement of a few LNA ODNs among other DNA-like nucleotide analogs within a mixmer ODN may provide a significant increase in single-nucleotide discrimination. Using novel nucleotide analogs such as LNAs which provide high affinity to an ODN may allow shortening of ODNs. It was demonstrated that relatively short ODNs (9mers and 11mers) against Ha-ras could be highly specific [22, 41]. In these short ODNs the effect of a single-nucleotide mismatch has far greater implications in the T_m as compared with a more conventional 20mer ODN. Indeed, when short LNA ODNs are used, almost absolute single-nucleotide specificity can be obtained in vitro [42].

RNA interference and allele-specific inhibition

Posttranscriptional gene silencing mediated by dsRNA represents an evolutionarily conserved cellular defence mechanism for controlling the expression of alien genes in protists, filamentous fungi, plants and animals [43–45]. It is believed that random integration of non-self genes (such as transposons) or viral infection causes production of dsRNA, which activates sequence-specific degradation of homologous single-stranded mRNA or viral genomic RNA, thereby preventing expression or replication of the foreign genetic material. In animal cells, the dsRNA-triggered silencing effect is referred to as RNA interference (RNAi) [46]. Especially in the nematode *Caenorhabditis elegans*, RNAi is used as a tool to study gene function [47]. Until recently, mammalian cells seemed not amendable to RNAi since the use of in vitro transcribed, long dsRNAs (>50 bp) as used in *C. elegans* led to activation of a global, sequence unspecific response resulting in blockage of initiation of protein synthesis and mRNA degradation [48]. However, Wianny et al. showed that RNAi might be used for the regulation of gene function in early mouse development [49]. More recently, it was reported by the group of Tuschl that duplexes of small 21-nt RNAs with 2-nt 3′ overhang (siRNA), specifically interfered with gene expression in mammalian cells without inducing the sequence-independent response of mammalian cells to long dsRNA [50, 51]. These short RNA duplexes are thought to resemble the processing products from the long dsRNA used in *C. elegans*. Furthermore, the same group showed that only the short single antisense RNA strand alone could initiate the same gene-silencing mechanism as siRNA [11]. In addition, prolonged stable suppression of gene expression by RNAi in mammalian cells was demonstrated [52]. siRNA possesses unique characteristics which imply that siRNA cannot only be used as a tool to study gene function, but might also be used as genotype-specific drug to mediate ASI. First, like antisense oligonucleotides, siRNA is highly sequence specific. Single-nucleotide mismatches abrogate the ability to suppress gene expression [52, 53]. In addition, Brummelkamp et al. [54] showed that it is possible to specifically inhibit the mutant *K-ras* allele using retroviral delivery of siRNA, while leaving the wild-type *K-ras* allele untouched, demonstrating that allele-specific targeting is possible with siRNA. Second, siRNA is active at very low concentrations as compared with antisense oligonucleotides [51]. Third, the small synthetic RNAs could be partially modified at the 2′-position of the ribose groups without affecting the efficacy [53]. These 2′ modifications might stabilize the molecule and possibly protect them from degradation by nucleases, as is extensively demonstrated with antisense ODNs. This might enable the use of siRNA as a stable pharmacological agent. In any case, 'naked' unmodified RNAi duplexes can be admistered to *C. elegans* nematodes by soaking them in solutions containing dsRNA, or by feeding them with bacteria carrying plasmids expressing dsRNA [55]. Finally, its was shown that siRNA could suppress gene expression in adult mice [56], demonstrating that dsRNA is quite stable and can work when given to a complex animal.

The success rate of finding a potent siRNA seems much higher in comparison with the success rate for finding an ODN with a good efficacy, which is a tedious process of trial and error. For siRNAs one out of three or four siRNAs seem to be effective. Of course, certain restrictions have been described in finding a working siRNA [53], but these seem minimal compared with the 'black box' of antisense ODN design. One must be careful with these conclusions: the field of siRNA is very young, and the history of antisense ODN development is marked with pitfalls. However, with our current knowledge it seems that the greater versatility of siRNa design as compared with antisense ODNs might allow for targeting more SNPs within an essential gene suitable for ASI than is possible with antisense ODNs. Most important, ASI has been demonstrated in tumors using a retroviral mediated siRNA delivery system [54]. These results suggest that the prospects for implementing ASI through siRNA are quite promising.

In summary, ASI is in theory an elegant and very powerfull approach to treating cancer. Since cancer cells cannot regain genetic sequences that they have lost during tumorigenesis, escape from this treatment is very difficult. However, since essential genes are targeted in healthy cells as well, it is very important to use highly selective drugs that discriminate between the two alleles. Oligonucleotide drugs may be designed that have these discriminatory powers. Several publications have shown that even classical phosphorothioate ODNs can provide single-nucleotide discrimination. We expect that new nucleotide chemistries such as LNAs might be applied to increase the selectivity and efficacy of the oligonucleotides. In addition, the recent discoveries of RNAi in mammalian cells has opened a new approach to developing nucleic acid-based genotype-specific drugs that may be used in ASI and other novel therapeutic approaches. These new and exciting developments in the development of nucleic acid-based therapeutics have prompted a renaissance for nucleic acids as drugs. Careful research in the next few years will determine whether this promise can be fullfilled and a potentially powerful therapeutic approach such as ASI can be utilized in the clinic.

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