DDT Vol. 7, No. 17 September 2002

Applications of antisense and siRNAs during preclinical drug development

James D. Thompson

A significantly greater number of candidate drug targets and compounds are now being generated during preclinical drug development. To date, however, such increases have not led to improvements in clinical success rates or reduced times to market. There is a need for better strategies to prioritize targets and drug candidates. Antisense and siRNA technologies offer exceptional speed and specificity to address this need. In particular, antisense and siRNAs are beginning to be used in combination with expression profiling to evaluate drug specificity and mechanism-of-action, aiding in the identification of better candidates earlier in the drug development process.

James D. Thompson
IMPACT Biosciences
4950 Arapahoe Ave.
Suite 100
Boulder, CO 80303, USA
tel: +1 303 415 2086
fax: +1 303 415 2500
e-mail: jthompson@impactbiosciences.com
Present address:
Ribozyme Pharmaceuticals
2950 Wilderness Place
Boulder, CO 80301, USA
tel: +1 720 406 2972
fax: +1 303 449 8829
e-mail: thompsj@rpi.com

The pharmaceutical industry relies on 'blockbuster' products for success. Blockbusters are necessary because most pharmaceutical companies commercialize, on average, only 1.4 new drugs per year. As such, these drugs must generate revenues sufficient to offset the high cost of development. According to the Tufts Center for the Study of Drug Development (http://csdd.tufts.edu), the average cost to develop a drug is US$ ~800 million. Such high costs result from long development times of 10–15 years and a high (90%) failure rate in clinical trials.

The industry is betting that advancements in genomics and HTS will increase success rates and reduce the time to market by producing better drug targets and drug candidates. Current drugs on the market target only ~500 different proteins and it is believed that there are thousands of new drug targets to be discovered [1]. As a result of improvements in HTS technologies during the past decade, 50-fold more drug candidates are now being screened per drug target. However, bottlenecks still exist in the drug discovery process, particularly at the stages of identifying appropriate targets for drug development, and prioritizing which drug candidates to extend into costly and time-consuming preclinical animal studies. Antisense and siRNA (small interfering RNA) technologies offer the speed and specificity required to address these bottlenecks. Effective antisense and siRNAs can be produced and validated in as little as four weeks. Because the sequences of most of the human genes are now known, specificity predictions can be performed *in silico*, facilitating the design of reagents capable of distinguishing between related targets. Such highly specific antisense or siRNA reagents can be used as 'gold standards' to evaluate the specificity of drug candidates. By incorporating antisense and siRNA technology effectively during preclinical development, companies can discover better drug targets faster and improve the decision process for choosing which drug candidates to progress down the development pipeline.

Antisense technology

Antisense oligonucleotides were first applied in cell culture more than 20 years ago [2]. Like most emerging technologies, antisense has experienced its share of growing pains [3,4]. However, antisense and functional genomics companies have made significant investments to optimize the technology for functional genomics applications and as therapeutics for treating human disease. At present, one antisense therapeutic is on the market (Vitravene™, Isis Pharmaceuticals; http://www.isip.com) and tens of candidates are in clinical trials ([5], and for an updated list of antisense reagents in clinical trials, see http://www.trilinkbiotech.com/newsite/Technical_Info/Articles/pdfs/antisense_oligo_primer.pdf).

There are two classes of antisense oligonucleotides: cleavers and blockers. As their names imply, the two classes are distinguished based on their mechanism-of-action. Cleavers

PII: S1359-6446(02)02410-8

bind by Watson–Crick base pairing to their targeted RNAs, leading to activation of intracellular nucleases that cleave the target RNA. The most common types of cleavers are those that activate the ubiquitous ribonuclease, RNase H [6–9]. There is an emerging class of antisense reagents that activates another ribonuclease, RNase L [10]. Blockers also bind to a target RNA via Watson–Crick base pairing, but inhibit protein translation via steric hindrance by acting as roadblocks to ribosomes. The most common blockers used are peptide nucleic acids [11], morpholinos [12], locked nucleic acids [13] and methylphosphonates [5].

Antisense companies distinguish themselves by the chemical modifications and associated intellectual property used to confer nuclease resistance to their antisense technology. Antisense cleaver technologies can be accessed through the GeneTrove division of Isis Pharmaceuticals (http://www.genetrove.com), Atugen AG (http://www.atugen.com) and Sequitur (http://www.sequiturinc.com). Peptide nucleic acids can be obtained through GeneTrove, morpholinos through Gene-Tools, LLC (http://www.gene-tools.com), locked nucleic acids through Proligo, LLC (http://www.proligo.com), and methylphosphonates through Trilink Biotechnologies (http://www.trilinkbiotech.com).

SiRNA technology

RNA interference, or RNAi, is a gene silencing mechanism originally discovered in simpler organisms, such as the nematode and fruit flies [14–17]. The pathway is activated by double stranded RNA (dsRNA). Once in the cell, the dsRNAs are processed into short, 21–22 nucleotide dsRNAs termed small interfering RNAs (siRNAs) that are used by the cell in a sequence-specific manner to recognize and destroy complementary RNAs [18,19]. The RNAi pathway has been exploited in simpler organisms to evaluate gene function by introducing dsRNAs that are specific to the targeted gene. There have been difficulties applying this strategy to cells from higher organisms, such as mammals, because of the presence of another dsRNA pathway that activates the interferon pathway [20]. However, Tuschl and co-workers recently developed a form of 21–22 nucleotide synthetic siRNAs that are too small to activate the interferon pathway in mammals but are of the correct structure to activate the RNAi pathway [21]. Thus, they and others have used the RNAi pathway successfully in mammalian cells to silence targeted genes.

A guide for the design and use of siRNAs is available from Tuschl's laboratory (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html), and in published form [22]. Synthetic siRNA reagents can be purchased from Dharmacon Research (http://www.dharmacon.com), Proligo, Pierce Chemical (http://www.perbio.com), Glen Research (http://www.glenres.com), ChemGenes (http://www.chemgenes.com) and Cruachem (http://www.Cruachem.com). In addition, Ambion (http://www.ambion.com) offers a kit to produce siRNAs enzymatically.

Antisense versus siRNAs

Antisense and siRNA technologies have many similarities. Mechanistically, both work at the post-transcriptional level to reduce the level of a target protein. In cell-culture applications, the delivery and dosing issues for antisense oligonucleotides and siRNAs are similar [23,24]. At present, commercially available antisense reagents are much more resistant to nucleases than current siRNAs, making antisense easier to apply to mammalian animal systems (see Refs [25,26]). Antisense is a more mature technology than siRNAs, and strict controls have been established for antisense experiments to avoid misleading results [27].

Antisense oligonucleotides accumulate in the nucleus [28] and therefore can be used to alter splicing of precursor mRNAs (Fig. 1) [29,30]. By contrast, siRNAs are thought to function in the cytoplasm [31] and probably cannot gain access to precursor mRNAs. It is estimated that up to half of all human genes produce more than one protein via alternative splicing [32,33]. Alternative splicing provides a mechanism to produce multiple proteins with different activities from a single gene [34]. Antisense cleavers (and potentially siRNAs) can be designed to inhibit expression of specific splice variants by targeting exon–exon junctions in mature mRNAs; antisense binders can be used to block splice acceptor sites (Fig. 1), providing a finer regulation of splicing events [29,30].

siRNAs have an advantage over antisense in that lower concentrations are needed to achieve levels of knockdown that are comparable to antisense reagents. Also, siRNAs can be expressed intracellularly from RNA polymerase III promoters [35–37]. This enables the production of stably expressing siRNA cell lines with sustained knockdown of a target and the potential to produce transgenic animals.

Why use oligonucleotide technologies for preclinical applications?

Antisense and siRNAs offer two main advantages over other gene-targeted technologies: speed and specificity [6–8]. It would be a daunting task for a chemist to produce, in just four weeks, three or more different compounds that are capable of inhibiting one specific target protein. Such rapid turnaround times and high specificity can be achieved with antisense and siRNA technologies. The short turnaround time enables the quick identification of good drug targets. The high specificity advantage of the technology

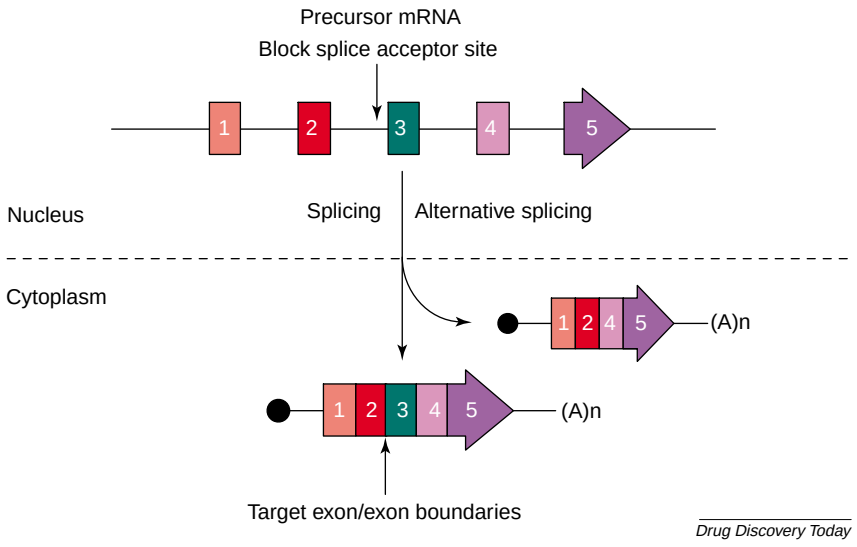

Figure 1. mRNA production and processing. Most mRNAs are generated from precursor RNAs that contain introns. Introns are spliced out of precursor RNAs in the nucleus during mRNA maturation. Antisense blockers can be designed to bind to splice donor sites to regulate splicing in the nucleus. Antisense cleavers and siRNAs can be designed to bind to exon–exon boundaries to block expression of certain splice variants.

can be exploited in conjunction with expression profiling to evaluate the specificity of drug candidates.

Speed: finding the right target

The sequences of most human genes are now known, and the next blockbuster drug targets are contained in this massive amount of information. However, both the US and European patent offices have raised the bar on gene patents. Specifically, the utility requirements are now stricter. Companies will not be rewarded simply for finding a new gene, but must demonstrate its significant and substantial use. This is where the speed advantage of antisense and siRNAs can add value. Those with expertise in oligonucleotide design and delivery can progress from raw sequence information to cellular gene function in as little as four weeks. Often, the same reagents that are used in cell culture studies can be extended to animal proof-of-principle studies. Such rapid turnaround times are possible because antisense and siRNAs can be designed *in silico* using sequence information alone. Selecting optimal reagents from the initial designs requires relatively simple cell-culture screening assays [8]. This is not the case for inhibitors such as antibodies, aptamers and small molecule drugs that typically take months to develop and require purified protein. Because of this speed advantage, antisense and siRNA technologies are ideal for rapidly screening candidate target genes, and for generating the functional data that is necessary to meet the stricter patent utility requirements.

Specificity: the advantage of targeting RNA

Many drug targets are members of large gene families encoding for proteins with similar 3D structures. Examples include the protein kinases, phosphatases and proteases. This similarity at the protein structural level makes it difficult to develop small-molecule drugs that are specific for a single family member. By contrast, antisense and siRNAs bind to the RNA coding for the protein, and RNAs from even highly similar proteins can be quite different.

COX-1 and COX-2 provide good examples of how two related targets can be distinguished by antisense (and probably siRNA) reagents. The COX proteins are the targets of aspirin, ibuprofen and other nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs exhibit their beneficial effects by inhibiting COX-2, but the first generation NSAIDs, like aspirin and ibuprofen, can produce unwanted side effects because they also inhibit COX-1 that is involved in various 'housekeeping' functions, such as homeostasis of the lining of the gut [38]. Consequently, developing drugs that are more specific for COX-2 has become a highly competitive, multi-billion dollar market. The 3D structures of the COX proteins are similar, which is why the first generation NSAIDs inhibit both isoforms. However, antisense and siRNA specificity is determined by the 2D sequence of the COX RNAs (excluding accessibility issues [8]). Figure 2 shows the similarity at the nucleotide sequence level between the different regions of the COX mRNAs. Note that the coding regions are only ~62% similar, whereas the untranslated regions are even less similar. It has been shown that antisense reagents can be optimized to distinguish as little as a single nucleotide difference in a targeted region, which corresponds to differentiating sequences that are ~95% identical [39]. Likewise, siRNAs have the potential for such high specificity [19]. As such, antisense reagents have been developed that are highly specific for one COX isoform over the other [40]. This specificity advantage can be applied to other targets, providing a gold standard to evaluate the specificity of drugs designed against such targets.

Antisense and siRNAs as model inhibitors to evaluate drug specificity

Almost all drugs exhibit unwanted side effects. The severity of such side effects can dictate market share, or cause

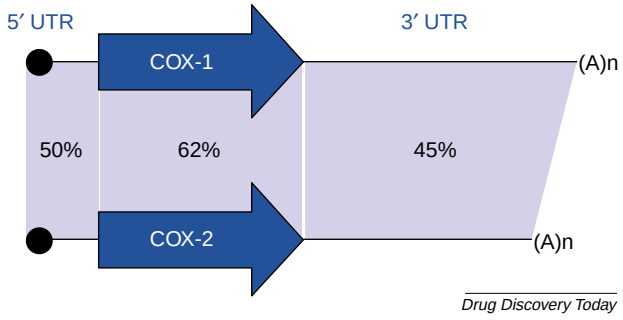

Figure 2. Comparison of COX-1 and COX-2 mRNA sequences. The 5′ untranslated regions (UTRs), coding regions and 3′ UTRs of the COX-1 (GenBank NM_000962) and COX-2 (GenBank NM_000963) mRNA sequences were compared using ALIGN [47–49] through the SDSC Biology Workbench (http://workbench.sdsc.edu/). Percentages indicate percent identity between the corresponding regions.

drugs to be pulled from clinical studies or from the market. Often, side effects arise because a drug interacts with other targets besides the intended target. Unfortunately, such side effects are not usually discovered until late-stage preclinical testing in animals or until clinical trials. Therefore, there is a need to detect the potential for side effects early, before significant resources are spent on sub-optimal candidates. There is an emerging and potentially powerful application of antisense and siRNA technologies to evaluate drug specificity and mechanism-of-action early during the cell culture phase of drug development. This new application uses the specificity advantage of antisense and siRNAs in conjunction with high content expression profiling endpoints.

Expression profiling is proving to be a powerful tool for diagnosing various cancers [41] and predicting which patients will respond to certain treatment regimens [42]. Similarly, profiling is beginning to be used in conjunction with antisense and siRNAs to evaluate effects of gene knockdown in cell culture. Sequitur has used expression profiling to evaluate how other genes are impacted when a target gene is inhibited by a specific antisense (see http://www.sequiturinc.com/genelink.htm). Atugen AG has used such antisense knockdown profiles as ‘gold standards’ for interpreting expression profiles generated from candidate drugs (see http://www.atugen.com/lead.htm).

The expression profile produced by a target-specific antisense or siRNA reagent can be visualized as the target pattern where the center bull’s-eye represents the intended drug target, and the concentric rings around the bull’s-eye represent the collection of genes that are impacted over time following inhibition of the intended target (Fig. 3). Candidate drugs that are perfectly specific to the intended

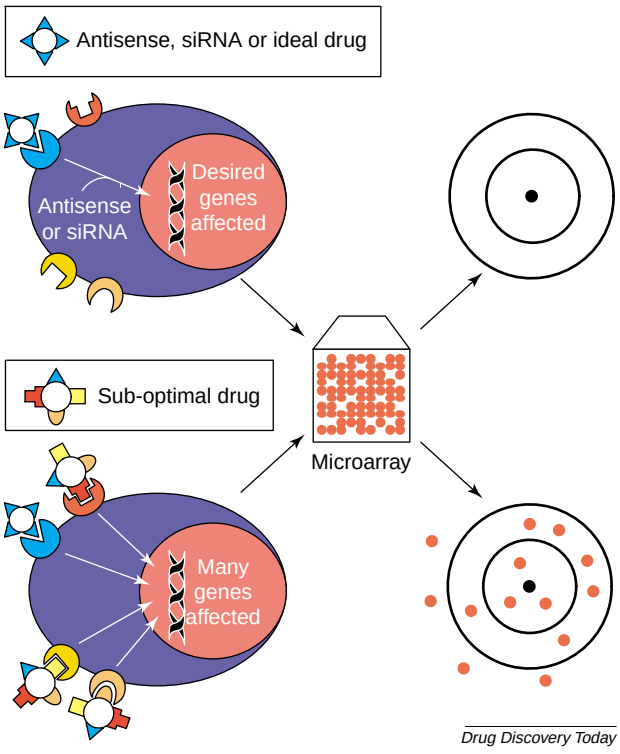

Figure 3. Evaluating drug specificity using antisense or siRNAs in combination with expression profiling. The specificity of a candidate drug can be evaluated by comparing model antisense or siRNA expression profiles with a matching expression profile from the candidate drug. A drug that is specific to the intended target should produce an expression profile similar to that of the antisense or siRNA. Sub-optimal drugs will interact with non-intended targets, represented as red dots that fall outside the concentric rings of the model antisense or siRNA profile.

target should produce a pattern identical to the antisense or siRNA profile. By contrast, non-specific drugs would affect the expression levels of other genes, producing the non-target effects in the drug expression profile illustrated in Fig. 3 as ‘hits’ that fall outside the concentric rings.

The non-target effects illustrated in Fig. 3 could represent something desirable, such as pathways that are important in other disease indications, thus leading to the identification of new indications for a drug. Alternatively, such non-target effects could represent something undesirable, such as a pathway that could cause an adverse side effect, or a pathway impacted by another drug leading to adverse drug–drug interactions. One strategy to determine if a pattern represents a potentially desirable or undesirable effect is to use a compendium of profiles to interpret expression profile patterns [43,44]. In the compendium approach, one compares a given profile to a library, or compendium, of profiles generated from known drugs and

targets. This approach has been used successfully in yeast to validate gene targets and to identify secondary drug target effects [43,44]. Expression profiling is most readily accomplished using microarrays designed to evaluate the status of many genes in a single experiment [45]. Arrays are now commercially available that contain probes for all of the 30,000+ human genes identified to date (e.g. GeneChip U133 set, Affymetrix; http://www.affymetrix.com). Libraries of drug profiles from mammalian cells are also commercially available from, for example, DrugMatrix (Incyte Genomics, http://www.incyte.com) and ToxExpress (Gene Logic, http://www.genelogic.com). In addition, hardware and software systems have been developed to manage and interpret expression-profiling data, for example, the Rosetta Resolver system (Rosetta Inpharmatics, http://www.rii.com).

When antisense and siRNAs don't make sense

Unlike classical drugs that bind to and inhibit the function of target proteins, antisense reagents bind to the RNAs that encode the target proteins, leading to a reduction or 'knockdown' of the protein. Because of the difference in mechanism-of-action between antisense–siRNAs and classical drugs, antisense and siRNAs might not represent the best model inhibitors for all targets. For example, removing a protein that is part of a protein complex could cause the complex to collapse, leading to a domino effect that might not be seen if the activity of the target protein is inhibited by a drug and the complex remains intact. For such targets, other inhibitors, such as nucleic acid aptamers [46] or antibodies that act at the protein level, could represent better model inhibitors (but less convenient because of the long development times).

Summary

Antisense and siRNA technologies can be used to address certain bottlenecks during preclinical drug development. Such technologies currently are being used to help identify the best drug targets for a given disease indication. An emerging and potentially powerful application of these technologies is now being explored to help prioritize drug candidates. In this application, highly specific antisense and siRNAs are used as model inhibitors in conjunction with expression profiling to evaluate the specificity of drug candidates to their intended targets and to identify potential side effects. Comparing antisense or siRNA model expression profiles to drug profiles could aid in prioritizing which drug candidates to extend into costly and time-consuming preclinical animal studies. This strategy could also be used during the medicinal chemistry phase of drug optimization to help develop compounds that are highly specific to their intended targets. Effective implementation of antisense and siRNA technology during preclinical drug development can increase the probability of success and accelerate the path to the clinic.

References

1 Drews, J. (2000) Drug Industry: a historical perspective. *Science* 287, 1960–1964
2 Zamecnik, P.C. and Stephenson, M.L. (1978) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. U. S. A.* 75, 280–284
3 Stein, C.A. (1995) Does antisense exist? *Nat. Med.* 1, 1119–1121
4 Wagner, R.W. (1995) The state of the art in antisense research. *Nat. Med.* 1, 1116–1118
5 Hogrefe, R.I. (1999) An antisense oligonucleotide primer. *Antisense Nucleic Acid Drug Dev.* 9, 351–357
6 Bennett, C.F. and Cowsert, L.M. (1999) Application of antisense oligonucleotides for gene functionalization and target validation. *Curr. Opin. Mol. Ther.* 1, 359–371
7 Bennett, C.F. (1998) Antisense oligonucleotides: is the glass half full or half empty? *Biochem. Pharmacol.* 55, 9–19
8 Baker, B.F. *et al.* (2001) Discovery and analysis of antisense oligonucleotide activity in cell culture. *Methods* 23, 191–198
9 Agrawal, S. (1999) Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides. *Biochim. Biophys. Acta* 1489, 53–68
10 Adah, S.A. *et al.* (2001) Chemistry and biochemistry of 2′,5′-oligoadenylate-based antisense strategy. *Curr. Med. Chem.* 8, 1189–1212
11 Nielsen, P.E. *et al.* (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Cancer Res.* 15, 5468–5473
12 Iversen, P.L. (2001) Phosphorodiamidate morpholino oligomers: favorable properties for sequence-specific gene inactivation. *Curr. Opin. Mol. Ther.* 3, 235–238
13 Orum, H. and Wengel, J. (2001) Locked nucleic acids: a promising molecular family for gene-function analysis and antisense drug development. *Curr. Opin. Mol. Ther.* 3, 239–243
14 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
15 Sharp, P.A. (2001) RNA interference 2001. *Genes Dev.* 15, 485–490
16 Plasterk, R.H.A. (2002) RNA silencing: the genome's immune system. *Science* 296, 1263–1265
17 Zamore, P.D. (2002) Ancient pathways programmed by small RNAs. *Science* 296, 1265–1269
18 Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952
19 Elbashir, S.M. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* 20, 6877–6888
20 Kumar, M. *et al.* (1998) Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol. Mol. Biol. Rev.* 62, 1415–1434
21 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
22 Elbashir, S.M. *et al.* (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26, 199–213
23 Hughes, M.D. *et al.* (2001) The cellular delivery of antisense oligonucleotides and ribozymes. *Drug Discov. Today* 6, 303–315
24 Lebedeva, I. *et al.* (2000) Cellular delivery of antisense oligonucleotides. *Eur. J. Pharm. Biopharm.* 50, 101–119
25 Fraser, G.L. *et al.* (2000) Antisense inhibition of delta-opioid receptor gene function *in vivo* by peptide nucleic acids. *Mol. Pharmacol.* 57, 725–731

26 Henry, S.P. *et al.* (1997) Toxicological and pharmacokinetic properties of chemically modified antisense oligonucleotide inhibitors of PKC-alpha and C-raf kinase. *Anticancer Drug Des.* 12, 409–420
27 Stein, C.A. (2001) The experimental use of antisense oligonucleotides: a guide for the perplexed. *J. Clin. Invest.* 108, 641–644
28 Fisher, T.L. *et al.* (1993) Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. *Nucleic Acids Res.* 21, 3857–3865
29 Sierakowska, H. *et al.* (2000) Antisense oligonucleotides and RNAs as modulators of pre-mRNA splicing. *Methods Enzymol.* 313, 506–521
30 Kole, R. and Sazani, P. (2001) Antisense effects in the cell nucleus: modification of splicing. *Curr. Opin. Mol. Ther.* 3, 229–234
31 Billy, E. *et al.* (2001) Specific interference with gene expression by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. U. S. A.* 98, 14428–14433
32 Venter, J.C. *et al.* (2001) The sequence of the human genome. *Science* 291, 1304–1351
33 Lander, E.S. *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* 409, 860–921
34 Pfeffer, U. *et al.* (1996) Alternative splicing of the estrogen receptor primary transcript normally occurs in estrogen receptor positive tissues and cell lines. *J. Steroid Biochem. Mol. Biol.* 56, 99–105
35 Miyagishi, M. and Taira, K. (2002) U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* 20, 497–500
36 Lee, N.S. *et al.* (2002) Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. *Nat. Biotechnol.* 20, 500–505
37 Paul, C.P. *et al.* (2002) Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* 20, 505–508
38 Simon, L.S. (2001) COX-2 inhibitors. Are there nonsteroidal anti-inflammatory drugs with a better safety profile? *Gastroenterol. Clin. North Am.* 30, 1011–1025
39 Giles, R.V. *et al.* (1995) Single base discrimination for ribonuclease H-dependent antisense effects within intact human leukaemia cells. *Nucleic Acids Res.* 23, 954–961
40 Yamada, R. *et al.* (2000) Selective inhibition of cyclooxygenase-2 with antisense oligodeoxynucleotide restricts induction of rat adjuvant-induced arthritis. *Biochem. Biophys. Res. Commun.* 269, 415–421
41 Issaq, H.J. (2002) The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. *Biochem. Biophys. Res. Commun.* 292, 587–592
42 van Veer, L.J. *et al.* (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415, 530–536
43 Hughes, T.R. *et al.* (2000) Functional discovery via a compendium of expression profiles. *Cell* 7, 109–126
44 Marton, M.J. *et al.* (1998) Drug target validation and identification of secondary drug target effects using DNA microarrays. *Nat. Med.* 4, 1293–1301
45 Brown, P.O. and Botstein, D. (1999) Exploring the new world of the genome with DNA microarrays. *Nat. Genet.* 21, 33–37
46 Brody, E.N. and Gold, L. (2000) Aptamers as therapeutic and diagnostic agents. *J. Biotechnol.* 74, 5–13
47 Myers, E.W. and Miller, W. (1988) Optimal alignments in linear space. *Comput. Appl. Biosci.* 4, 11–17
48 Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U. S. A.* 85, 2444–2448
49 Pearson, W.R. (1990) Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.* 183, 63–98