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Chemical name

BSU1051: 2,6-diamido anthraquinone

Elucidating cell signaling mechanisms using antisense technology

Erich Koller, William A. Gaarde and Brett P. Monia

Many diseases result from defects in cell signaling. Achieving an in-depth understanding of the complex mechanisms by which cells transduce extracellular signals into cellular responses in both normal and diseased systems is a crucial step in the discovery of more effective drugs to treat human diseases. Traditional approaches for studying cell signaling have some limitations. Antisense oligonucleotides represent a novel approach for studying signal transduction processes that offers significant advantages in terms of specificity and versatility. This article reviews the opportunities that antisense oligonucleotides offer for the study of signal transduction pathways and identification of inhibitors of these pathways for drug development.

Experimental approaches are essential for determining the biological functions of cellular gene products. However, few methods offer acceptable specificity in a time- and cost-effective manner; consequently, progress in identifying the function of the numerous genes that are implicated in disease pathology has been slow. Furthermore, after an essential role for a particular gene product in a disease process has been demonstrated, the emergence of novel therapies has also been slow, because most approaches that are useful for determining gene function in preclinical models are unsuitable pharmaceutical agents for treatment in humans.

The need for novel methods to identify the function of specific gene products can be best exemplified in the field of signal transduction. Virtually every signal transduction pathway contains components with multiple isoforms. This complexity is compounded because individual signaling pathways often overlap and ‘cross-talk’ with other pathways, which results in either modulation or redundancy of pathways. Thus, there is a great need for novel approaches to discriminate, rapidly and selectively, the biological function of structurally and functionally homologous gene products.

Traditional approaches for studying cell signaling

Historically, pharmacological inhibitors of cell signaling pathways have been identified serendipitously. For example, although aspirin is widely used in the treatment of inflammation and pain, it has taken a long time to attain a basic understanding of its mechanism of action [e.g. inhibition of prostaglandin synthesis or IκB kinase β (IKKβ)]¹. To develop inhibitors of signal transduction pathways, the proteins involved in these processes must be identified and their functions defined by selective and efficient modulation of their activity. It is generally believed that this process, sometimes referred

to as target validation, will result in the rational discovery of drugs with more attractive therapeutic indices.

The approaches that have traditionally been used for the study of cell signaling pathways (Fig. 1) can be classified into three major areas: (1) gene knockout systems, in which the function of a particular protein is studied by disrupting the function of the gene that encodes the protein; (2) over-expression systems, in which the function of a particular protein, or a derivative thereof (e.g. a dominant-negative mutant), is studied by expressing it at unusually high levels; and (3) small-molecule, peptide or antibody inhibitors, in which inhibitor activity is correlated with the function of a specific protein or cell pathway, or with a specific phenotype (e.g. apoptosis). Each approach has furthered our understanding of cell signaling pathways considerably. However, for several reasons, these approaches have limited use (Table 1).

The antisense concept

Antisense oligonucleotides represent a new paradigm for the discovery of potent and selective drugs with fewer side-effects. The antisense concept is based on an understanding of nucleic acid structure and function, and depends on Watson–Crick hybridization mechanisms². Antisense oligonucleotides are designed to inhibit the translation of RNA specifically by binding with high affinity and selectivity to their RNA targets (Fig. 1); they exert their inhibitory effects on mRNA function by several mechanisms, including inhibition of splicing, inhibition of protein translation by disrupting ribosome assembly, and most commonly, through the utilization of endogenous RNase H enzymes, which recognize the mRNA–oligonucleotide duplex and degrade the mRNA strand^{2,3}. The specificity of antisense technology allows

E. Koller,
Senior Scientist,
E-mail: ekoller@
isisph.com

W.A. Gaarde,
Senior Scientist,
E-mail: bgaarde@
isisph.com

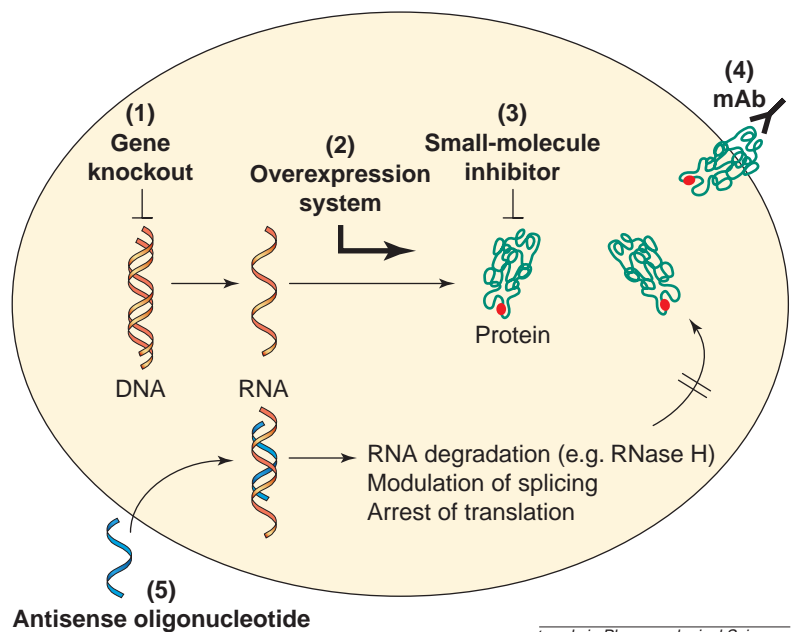
and
B.P. Monia,
Executive Director,
Department of
Molecular and
Cellular Pharmacology,
Isis Pharmaceuticals,
2292 Faraday Avenue,
Carlsbad,
CA 92008, USA.
E-mail: bmonia@
isisph.com

investigation of the detailed mechanisms of signal transduction processes, which often comprise families of highly homologous proteins.

Antisense specificity

Specificity and simplicity are two major advantages of the antisense approach for discovering novel inhibitors of protein expression. Because antisense oligonucleotides act by targeting virtually any region (including non-translated sequences) within a pre-mRNA or mRNA, and as a result of the degeneracy of the genetic code, it is relatively straightforward to identify an antisense oligonucleotide that inhibits a member of a multigene family in a highly specific manner⁴. In addition, antisense oligonucleotides can be used to determine the function of related proteins that are produced by alternative splicing of a common gene by modulating the splicing process³. Furthermore, because the intended mechanism of action of antisense oligonucleotides is rational and well understood, it is relatively straightforward to satisfy the criteria for proving an antisense mechanism of action by directly measuring suppression of target mRNA and protein expression, and by using non-antisense designed ‘control’ oligonucleotides (e.g. scrambled sequences, mismatched sequences, etc.)², which decrease hybridization affinity, thereby attenuating the pharmacological effect of the antisense oligonucleotide^{2,3}. In addition, multiple antisense inhibitors that are of relatively equal potency for suppressing target expression can be used to demonstrate similar phenotypic effects.

Antisense oligonucleotide approaches have been employed successfully in functional studies to suppress expression of multiple isoforms of proteins simultaneously or individual isoforms separately⁵ (Table 2). An example of this is the Jun N-terminal kinase (JNK) family of mitogen-activated protein (MAP) kinases. The diligent search for selective small-molecule inhibitors of JNK activity by pharmaceutical companies has been largely unsuccessful. However, antisense oligonucleotides have been successfully employed to inhibit JNK protein expression selectively without affecting the expression of other kinases, and have been used to demonstrate unique functions for JNK isoforms in cell signaling pathways^{4,6,7} (Fig. 2). For example, antisense oligonucleotides that target JNK2, but not JNK1, have been shown to suppress the expression of cell adhesion molecules in response to tumor necrosis factor α (TNF- α) in endothelial cells⁴.



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Fig. 1. Experimental approaches that are currently used to modulate the activity or expression levels of a particular signaling molecule to determine the function of cell signaling molecules include: (1) gene knockout; (2) overexpression systems; (3) small-molecule inhibitors; (4) monoclonal antibodies (mAbs); and (5) antisense technology. The advantages and disadvantages for each approach are described in the text. Antisense technology uses chemically modified oligonucleotides that are designed to hybridize with target RNA sequences through a Watson–Crick hybridization mechanism that involves the formation of adenine–thymine (A–T) and guanosine–cytosine (G–C) base pairs.

Specificity problems of traditional methods

Small-molecule inhibitors

The routine discovery of suitable small-molecule inhibitors is inefficient in part because of specificity problems. Proteins that catalyse similar chemical reactions (e.g. phosphotransfer reactions for protein kinases) are highly conserved in areas that are most vulnerable for small-molecule intervention. However, there has been some success using this approach. Examples include inhibitors of p38 MAP kinase, MEK1,2 [MAP/extracellular regulated kinase (ERK) 1,2] and protein farnesyltransferase. These inhibitors have been valuable tools for investigating the function of specific cell signaling molecules and have resulted in the initiation of some clinical trials⁸. However, the specificity of these inhibitors has been questioned. For example, SB203580, in addition to inhibiting p38, has been shown to inhibit the activity of cyclooxygenases and other MAP kinases⁹. Similarly, recent reports have suggested

Table 1. A comparison of different experimental approaches for modulating the function of cell signaling molecules

Method	Versatility	Specificity	Required resources	Cost	Probability of success	Potential for drug development
Overexpression systems	Low to moderate	Moderate	Moderate	Low	Moderate	Low
Gene knockouts (mammalian)	High	High	High	High	Moderate	None
Small-molecule inhibitors	Low	Low	High	High	Low	Yes
Monoclonal antibodies	Low	High	Moderate	Moderate	Moderate	Yes
Antisense oligonucleotides	High	High	Low to moderate	Low to moderate	High	Yes

Table 2. Examples in which antisense technology has been employed to determine protein function in signal transduction pathways

ASO target ^a	Cell type	Biological system and endpoints ^b	Refs
ERK1,2	Cardiac myocytes	ANF response, sarcomerogenesis	5
	Vascular smooth muscle cells	DNA synthesis, p90 RSK activation	26
JNK1,2	Erythroleukemia	Apoptosis	27
	Lung tumor	Proliferation	7
	Endothelial cells	CAM expression (TNF- α)	4
	Glioblastoma	Proliferation	6
RAS (Ha, Ki, N)	Renal epithelial cells	Apoptosis	28
	Endothelial cells	CAM expression (TNF- α)	4
	Bladder carcinoma, fibroblasts	Proliferation	25
RAF	Endothelial cells	CAM expression (TNF)	4
	Smooth muscle cells	Proliferation	29
	Fibroblasts	ERK activation	4
MAP kinase phosphatase	Vascular smooth muscle cells	ERK activation	30
PI 3-kinase	Ovary cells, insulin responsive CHO (GRC-LR173)	DNA synthesis (insulin)	31
PKC α	Lung tumor	CAM expression	4
PKC ζ	Vascular smooth muscle cells	ERK activation	4
PKC ϵ	Endothelial cells	ERK activation	4
RhoA	Lung tumor	JNK activation	32
JAK2	Monocytes	15-LO activation	33
TYK2	Monocytes	15-LO activation	33
P125 ^{FAK}	Tumor (multiple)	Apoptosis	4
G α_2	Neuronal	Ca ²⁺ mobilization	34
G α_3	Insulinoma	Adenylate cyclase activation	35
BCL2	Lung tumor	Apoptosis	36
A1, BCLX	Endothelial cells	Apoptosis	37
PP5	Bladder carcinoma, fibroblasts	p53 activation	38
MDM2	Tumor (multiple)	p53 activation	39
Survivin	Cervical tumor	Polyploidy	40

^aTarget indicates the protein encoded by the intended RNA target (typically mRNA) for which the ASO was designed to suppress.

^bSee references for explanation.

Abbreviations: ANF, atrial natriuretic factor; ASO, antisense oligonucleotide; CAM, cell adhesion molecule; CHO, Chinese hamster ovary cells; ERK, extracellular regulated kinase; JAK, Janus-activated kinase; JNK, Jun N-terminal kinase; 15-LO, 15-lipoxygenase; MAP, mitogen-activated protein; p90 RSK, p90 ribosomal S6 kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PP5, serine/threonine protein phosphatase 5; TNF- α , tumor necrosis factor α ; TYK2, tyrosine kinase 2.

that RAS is not the primary target underlying the anti-tumor activity displayed by farnesyltransferase inhibitors⁸.

Overexpression systems

Determination of the biological function of a protein by overexpression of proteins, and mutants thereof, to non-physiological levels has only limited use for various reasons. For example, protein overexpression can alter the quality and quantity of protein-protein interactions because these interactions are governed largely by protein concentration. In addition, protein overexpression can alter intracellular protein localization.

Dominant-negative mutant proteins (e.g. 'kinase-dead' or 'substrate-dead') often maintain their ability to interact with other proteins because the mutation can be distinct from sites involving additional protein-protein interactions (e.g. scaffold functions). Because activities of this nature are known to be critical for the normal function of many signaling pathways, approaches that address only catalytic functions for signaling molecules might produce incomplete, or possibly inaccurate,

descriptions of protein function. For example, transcription factors belonging to a specific family [e.g. FOS, JUN or nuclear factor κ B (NF- κ B) families] often interact with factors within that same family, as well as with transcription factors from other families. These interactions, which can be phosphorylation independent, can greatly affect the functional response. Thus, dominant-negative mutants of transcription factors, which cannot be phosphorylated, are still free to interact and dimerize with other transcription factor subunits, thereby exerting a titration effect on the subunit pool and potentially altering the biological response in a way that is not directly related to the function of the protein.

Overexpressing proteins sometimes produces data that conflict directly with other results. For example, cells from knock-out mice that lack the signaling protein, RIP1 (receptor-interacting protein 1) fail to activate NF- κ B in response to TNF- α , whereas JNK activation is intact. This is in contrast to overexpression results that suggest RIP1 is required in the activation of both JNK and NF- κ B (Ref. 10). Antisense oligonucleotides have the potential to clarify confusing results of this nature.

Antisense versatility and efficiency

Lack of versatility limits the usefulness of many approaches. Small-molecule inhibitors have demonstrated their use as signal transduction inhibitors against receptor proteins and proteins that possess intrinsic enzymatic activity. However, signaling pathways also rely on adaptor proteins, scaffold proteins and transcription factors. These proteins typically function through specific protein-protein interactions that involve points of spatial contact over broad structural areas, making the possibility of small-molecule intervention almost impossible. Alternatively, the use of monoclonal antibodies (mAbs) as signal transduction inhibitors is primarily limited to cell-surface proteins and secreted proteins because of their inefficient delivery into cells. Antisense technology does not have these limitations. Antisense oligonucleotides act by targeting RNA and not protein. Therefore, they can inhibit the expression of virtually any type of protein regardless of its function. This includes proteins that are sometimes relatively easy to inhibit using traditional methods (e.g. enzymes and receptors) as well as proteins for which it is often difficult to obtain inhibitors (e.g. adaptor proteins, structural proteins and transcription factors) (Table 2). In addition, the level of target inhibition can be easily titrated by altering the antisense oligonucleotide dose or concentration, which is an advantage compared with gene knockouts, in which no protein is expressed. This is important because different functional responses, including toxicity, will probably be affected by the level of inhibition that is produced. The level of inhibition of a key signaling protein might determine how many downstream pathways are impacted and to what extent.

Full-length gene sequence information is not required to obtain effective antisense oligonucleotide inhibitors. This is particularly important for determining the function of molecules for which only partial cDNA sequences (e.g. expressed sequence tags) can be found in genomic databases. Even with a partial gene sequence, antisense oligonucleotides can

be rapidly designed, synthesized and tested in cell culture to determine if the target gene is inhibited, making it possible to test hypotheses rapidly for addressing signal transduction mechanisms.

Finally, antisense technology is a very efficient approach for discovering high-quality inhibitors of gene function. Fewer resources are required for this approach than by any other drug discovery method. High-quality inhibitors are discovered typically within weeks and, as mentioned above, all types of proteins are theoretically amenable to the approach. Identification of small-molecule inhibitors is still slow and time-consuming, although it has been facilitated with the development of high-throughput screening procedures in which thousands of small molecules are tested for activity against a specific reaction or phenotype. Furthermore, small-molecule compounds typically require follow-up structure-activity studies to optimize their activity.

Antisense oligonucleotides as drug candidates

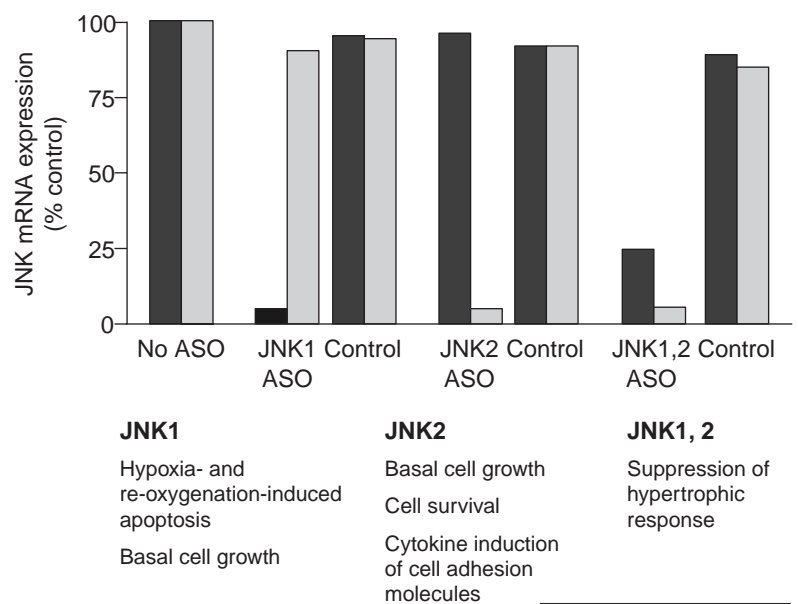
Despite the progress in the identification and implication of specific gene products as causal factors in human diseases, the development of therapeutics that specifically target these gene products has been slow. A major advantage of antisense technology for the study of cell signaling pathways and the clinical development of novel signal transduction inhibitors is the potential ability to use the lead antisense oligonucleotide that was identified in cell culture studies in animal models and, ultimately, in humans.

Recent publications have demonstrated that antisense oligonucleotides identified as inhibitors of gene expression in cell-based assays, are also effective in a wide range of animal disease models (e.g. models of cardiovascular disease, inflammatory disease, organ transplant, neurological disorders and cancer) in a manner that is highly consistent with an *in vivo* antisense mechanism of action¹¹⁻¹³. The number of clinical trials that have been initiated to evaluate antisense drugs for human therapeutics is growing at an impressive rate⁴ (Table 3). Moreover, the recent approval of the first antisense oligonucleotide therapeutic (Vitravene) by the Food and Drug Administration (FDA) for the treatment of cytomegalovirus-induced retinitis demonstrates that antisense oligonucleotides have good potential as therapeutic agents for treating human diseases¹⁴. Thus, antisense technology offers both the selectivity and versatility for the functional validation of cell signaling molecules, and a novel therapeutic approach for the treatment of human diseases in the clinic.

Potential limitations of antisense oligonucleotides

Protein half-life

Because antisense oligonucleotides target RNA and not protein, biological consequences that result from the inhibition of a particular gene product is dependent on the normal decay rate (i.e. half-life) of the encoded protein product. This is usually not a significant concern because most proteins possess a half-life of a few hours to a day. However, some proteins are very long-lived and might require up to several days to reduce their steady-state levels sufficiently. Obviously, being forced to wait for such extended periods might have certain



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Fig. 2. Use of antisense oligonucleotides (ASOs) to demonstrate specific roles (lower panel) for Jun N-terminal kinase (JNK) isoforms in cellular responses. Analysis of JNK1 and JNK2 mRNA levels (upper panel) was performed by northern blot analysis following treatment of human umbilical vein endothelial cells (HUVECs) with antisense oligonucleotides (JNK1, black; JNK2, grey). JNK mRNA levels, normalized to glucose-3-phosphate dehydrogenase (G3PDH) mRNA levels, were quantitated by phosphorimage analysis. Specific antisense oligonucleotides suppress JNK1 and JNK2 mRNA levels in an isoform-specific manner, and suppress both JNK protein levels and JNK activity. Suppression of JNK1 gene expression has been shown to inhibit proliferation of tumor cells^{6,23}, whereas suppression of JNK2 gene expression has been shown to decrease proliferation and cell survival of tumor cells *in vitro* and to inhibit tumor growth in animals^{6,7}. In addition, suppression of JNK2 expression has been shown to inhibit tumor necrosis factor α (TNF- α)-induced expression of cell adhesion molecules⁴.

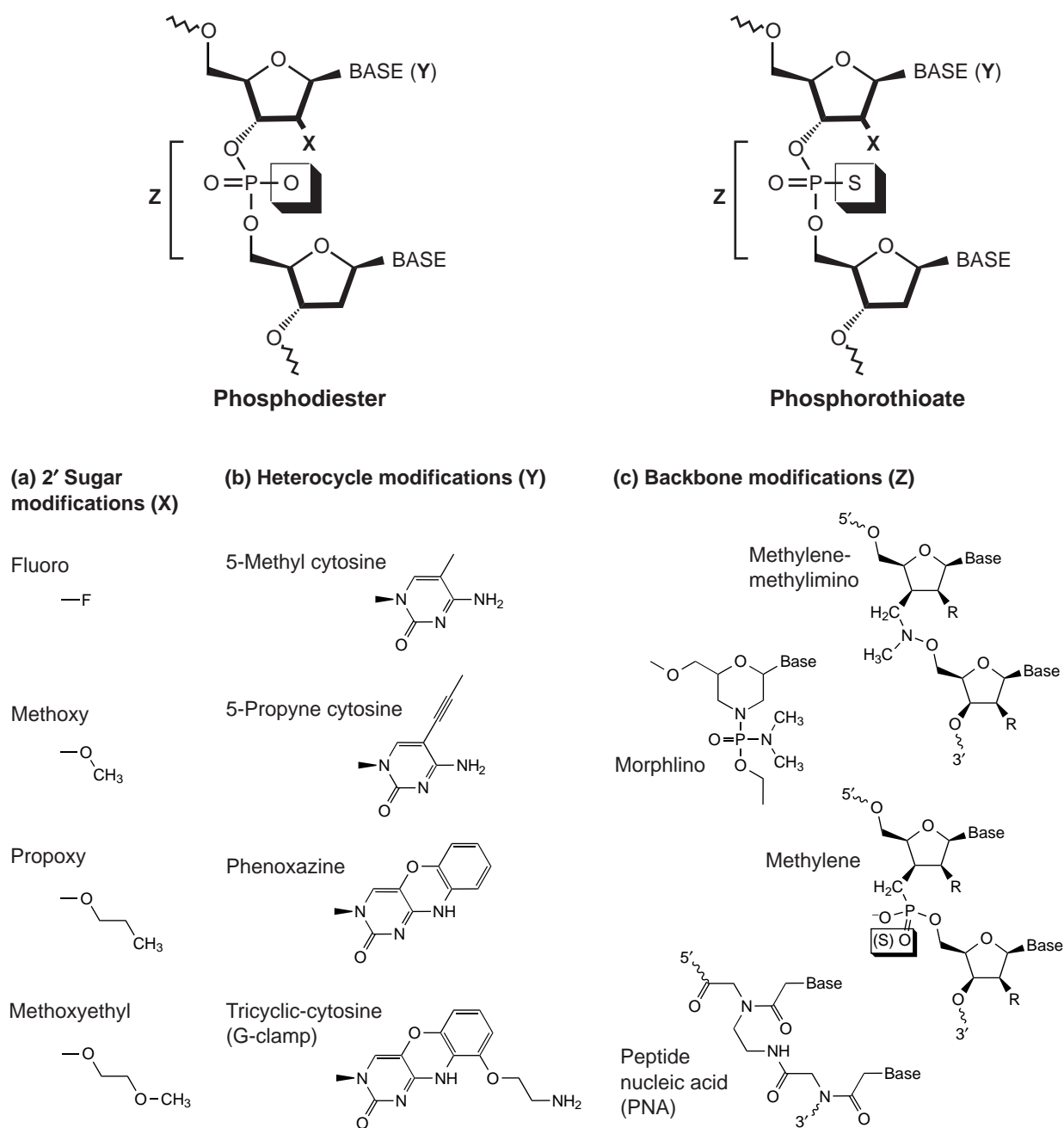
practical limitations and might impact the biological responses observed as a result of inhibiting gene expression. Therefore, the most attractive antisense targets for determining gene function are those that are either inducible or encode proteins with a short to moderate half-life.

Table 3. Antisense oligonucleotides currently in clinical trials or on the market

Compound	Protein target	Indication	Sponsoring company	Development phase
Vitravene (ISIS2292) ^a	CMV IE2	CMV retinitis	Isis/Ciba vision	Approved
ISIS2302	ICAM-1	Crohn's disease, organ transplant, psoriasis	Isis	Phase II
ISIS3521	Protein kinase C α	Cancer	Isis	Phase II
ISIS5132	RAF kinase	Cancer	Isis	Phase II
G3139	BCL2	Cancer	Genta	Phase II
INX3280	MYC	Restenosis	INEX	Phase II
GEM132	CMV UL36	CMV retinitis	Hybridon	Phase I
ISIS2503	Ha-RAS	Cancer	Isis	Phase II
ISIS13312	CMV IE2	CMV retinitis	Isis	Phase I
GEM92	HIV	AIDS	Hybridon	Phase I
GEM230	Protein kinase A	Cancer	Hybridon	Phase I

^aVitravene (Fomivirsin, ISIS2922) has been approved for the second-line treatment of cytomegalovirus (CMV) retinitis in patients with AIDS who are intolerant of or unresponsive to previous treatment(s) for the disease¹⁴. All drug compounds are phosphorothioate oligodeoxynucleotides except ISIS13312 (2'-methoxyethyl), GEM92 (2'-methoxy) and GEM230 (2'-methoxy), which contain 'second-generation' 2'-sugar modifications.

Abbreviations: ICAM-1, intercellular adhesion molecule 1; IE2, immediate early gene 2.



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Fig. 3. Structures of chemical modifications that are employed in antisense technology. Phosphodiester oligodeoxynucleotides ('plain DNA') are not useful in antisense technology because of their inherent susceptibility to nuclease degradation. Chemical modifications involving substitution within the **(a)** sugar, **(b)** heterocycle (base) or **(c)** backbone substituents of DNA were designed primarily to reduce nuclease sensitivity, improve affinity for RNA hybridization, or both. Some of these modifications also provide pharmacokinetic and toxicological advantages. Phosphorothioate oligodeoxynucleotides are sometimes referred to as 'first-generation' antisense oligonucleotides whereas oligonucleotides containing other modifications are referred to as 'second-generation'. (See references for a description of the chemical and biological properties of the modifications indicated^{17,24,25}.)

Post-translational modification

An additional limitation to antisense technology that is inherent to its mechanism of action is the potential inability to inhibit gene function for proteins that are regulated post-translationally. If a cell regulates the steady-state levels of a particular protein product by adjusting its protein half-life relative to mRNA production, approaches that inhibit RNA production might be unable to reduce steady-state protein levels effectively. Although such an outcome has yet to be

reported for an antisense experiment, it demonstrates the importance of considering the possible mechanisms by which cells might compensate post-translationally upon inhibition of mRNA function.

Immunostimulatory side-effects

The first chemical generation of antisense analogs is the phosphorothioates, in which one of the non-bridging phosphoryl oxygens of DNA is substituted with sulfur (Fig. 3). This

relatively simple modification results in dramatic improvements in nuclease stability and the *in vitro* and *in vivo* pharmacokinetics of oligonucleotides¹⁵. However, phosphorothioates have been reported to possess immunostimulatory effects in animals and humans^{11,16}. To circumvent these problems, newer oligonucleotide modifications have been identified that reduce or eliminate the nonspecific activities associated with phosphorothioate modifications^{17–20}. Typically, this is accomplished using chemistries that leave the natural phosphodiester DNA backbone intact but modify the 2' sugar position, producing a molecule with increased affinity for RNA and sufficient stability against nuclease degradation. However, backbone and heterocycle modifications are also attractive second-generation oligonucleotide chemistries that offer superior properties for functional gene studies. Thus, when possible, it is prudent to use newer oligonucleotide chemistries that display an improved activity profile compared with phosphorothioate antisense oligonucleotides.

Empirical approach for antisense oligonucleotide discovery

The selection of the optimal antisense sequence for inhibiting a particular mRNA still requires a fairly empirical approach in which multiple oligonucleotides must be evaluated because of inaccessibility of certain hybridization sites in target transcripts⁴. This restriction is a result of the inability to predict the optimal hybridization sites within a pre-mRNA sequence that stems, in part, from our inability to predict RNA structure in cells accurately. Thus, the practical aspects of synthesizing and testing a series of oligonucleotides should be considered before embarking on an antisense approach.

Cellular delivery

The conditions for delivering antisense oligonucleotides into cells efficiently must be considered carefully when using antisense oligonucleotides. Although the efficient delivery of antisense oligonucleotides into cells *in vitro* is generally straightforward using cationic lipid transfection approaches²¹, some cell types can be problematic and might require alternative delivery approaches. For example, other types of delivery vehicles (e.g. dendrimers) are sometimes better suited for delivering antisense oligonucleotides into certain cell types, and electroporation delivery of antisense oligonucleotides has frequently been used with success²². It is also worth noting that primary cell types in cell culture can often take up antisense oligonucleotides efficiently without the need for transfection.

Parenteral administration of saline-formulated antisense oligonucleotides has been shown to inhibit target gene expression in a variety of different tissue types and cell types in animals in a manner that is highly consistent with an antisense mechanism of action^{11,12}. However, the *in vivo* pharmacokinetics of antisense oligonucleotides is complicated and, not surprisingly, several cell types do not take up antisense oligonucleotides *in vivo* sufficiently enough to produce robust antisense effects. Approaches to improve *in vivo* delivery to these low-affinity cell types are currently being pursued using high-affinity antisense oligonucleotide chemistries (Fig. 3), as well as formulations. Oral delivery of antisense oligonucleotides *in vivo* is an additional hurdle for antisense technology to over-

come for certain drug applications. However, a great deal of progress has been achieved in recent years in this area, again through the use of novel antisense oligonucleotide modifications and formulations. Obviously, at present, oral bioavailability is an area in which small-molecule drugs possess a distinct advantage over antisense as drug candidates.

Concluding remarks

Recent progress in antisense technology demonstrates that antisense inhibitors can be successfully employed for abrogating the function of specific gene products *in vitro* and *in vivo*. The antisense approach offers many distinct and significant advantages over more traditional approaches for the study of cell signaling pathways and the development of novel signal transduction inhibitors. Thus, this approach provides, at the present time, significant value for studying both the function of specific gene products and the discovery of novel therapeutic agents to treat human diseases. To further advance antisense technology, important goals for the future include more efficient delivery to certain cell types *in vivo* and achieving sufficient oral bioavailability for antisense drugs in the clinic.

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Chemical name

SB203580: 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole

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