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AEG-35156

Antisense Targeting XIAP
Apoptosis Inducer
Oncolytic

GEM-640

Fully phosphorothioated second-generation 19-mer mixed-backbone antisense oligonucleotide (ASO) consisting of an 11-nucleotide DNA core flanked at the 3' and 5' ends by four 2'-*O*-methyl RNA residues, targeting the X-linked inhibitor of apoptosis protein (XIAP)

EN-351185

ABSTRACT

AEG-35156 is a fully phosphorothioated second-generation 19-mer mixed-backbone antisense oligonucleotide (ASO) consisting of an 11-nucleotide DNA core flanked at the 3' and 5' ends by four 2'-O-methyl RNA residues. The ASO was rationally designed to target the X-linked inhibitor of apoptosis protein (XIAP). XIAP, which is a member of the family of IAPs, has been shown to have an increasingly complex role in cancer. Although considerable evidence now supports XIAP as a validated target in anticancer drug design, the efficacy of antisense as a therapeutic approach remains to be proven clinically, especially in the treatment of cancer. Preclinically, AEG-35156 exhibits high potency and selectivity for its target and exerts broad-spectrum antitumor activity against a panel of human cancer xenografts. It also combines synergistically with conventional cytotoxic drugs to induce long-term tumor regression. The ASO entered its first phase I clinical trial in cancer patients in 2004, where it was administered as either a 7- or 3-day continuous infusion on a 21-day treatment cycle. Several other phase I/II trials have also been initiated exploring different administration schedules and the effect of combination with chemotherapy in more focused disease groups. Pharmacodynamic and biomarker studies played an important role in these clinical trials. Evidence has now accrued in cancer patients for target knockdown in surrogate tissues, leading to the induction of apoptosis, and clinical responses have been reported.

BACKGROUND

Failure to enter into apoptosis —a highly conserved program of cell death (1, 2)— is one of the classic hallmarks of cancer (3), enabling cells to survive and proliferate in the hostile environment of a tumor while continuing to accrue genetic instability (4). Thus, the signaling pathways and homeostatic mechanisms that regulate programmed cell death have themselves emerged as important targets in the design and discovery of new anticancer agents (5). In this review, we focus on one of the key elements that negatively regulates the apoptotic cascade, the X-linked inhibitor of apoptosis (XIAP), and a recently developed therapeutic that targets this protein, the antisense oligonucleotide (ASO) AEG-35156.

The inhibitors of apoptosis proteins

The inhibitors of apoptosis proteins (IAPs) are a multifunctional family of structurally related proteins that perform crucial roles within the cell, not only in the suppression of programmed cell death, but also in the regulation of signal transduction pathways and the control of cellular proliferation (6-8). They are the only known endogenous inhibitors of caspases, a class of cysteine-aspartyl proteases activated and recruited to execute the terminal phase of apoptosis (9-12). Due to their pivotal position in the apoptotic cascade, the IAPs are subject to tight and complex regulation at the gene, protein and functional levels. Proposed control mechanisms include phosphorylation, ubiquitination, protein/protein interactions, proteosomal processing and functional inhibition by derepressor proteins, including Smac/Diablo and OMI/HTRA2 (13, 14).

IAPs were initially described in baculoviruses in 1993 – where the product of the *Cydia pomonella IAP* gene was shown to act as a pan-caspase inhibitor (15). Two years later, the first mammalian IAP, neuronal apoptosis inhibitory protein (NAIP), was discovered by positional cloning during attempts to identify the causative agent in spinal muscular atrophy (16). Between 1995 and 2001, a further seven members were added to the human gene pool: X-linked inhibitor of apoptosis protein (*XIAP*) (17, 18), cellular IAP1 (*BIRC2*) and IAP2 (*BIRC3*) (19, 20), testis-specific IAP (*BIRC8*) (21), livin (*BIRC7*) and survivin (*BIRC5*) (22), and the ubiquitin-conjugating BIR domain enzyme apollon (*BIRC6*, *BRUCE*) (23) (see Fig. 1).

The human homologues are characterized structurally by the presence of the highly conserved baculovirus IAP repeat (BIR) domains (see Fig. 1) (24). Each different BIR domain is believed to be responsible for an independent IAP function. The BIR3 domain interacts selectively with caspase-9, resulting in enzyme inhibition (25, 26). Caspase-9 is the main effector in the intrinsic pathway of apoptosis, mediated via the mitochondria and normally activated in response to acute cellular stress stimuli (27). While originally it was believed that BIR2 was solely responsible for inhibition of the executioner caspases caspase-3 and

Jeffrey Cummings[1]*, Emma Dean[1, 2], Tim Ward[1], Malcolm Ranson[2], Caroline Dive[1].
[1]Clinical and Experimental Pharmacology, Paterson Institute for Cancer Research, University of Manchester, Wilmslow Road, Manchester, M20 4BX, U.K.; [2]Department of Medical Oncology, University of Manchester, Christie Hospital NHS Foundation Trust, Wilmslow Road, Manchester, M20 4BX, U.K. *Correspondence: jcummings@picr.man.ac.uk

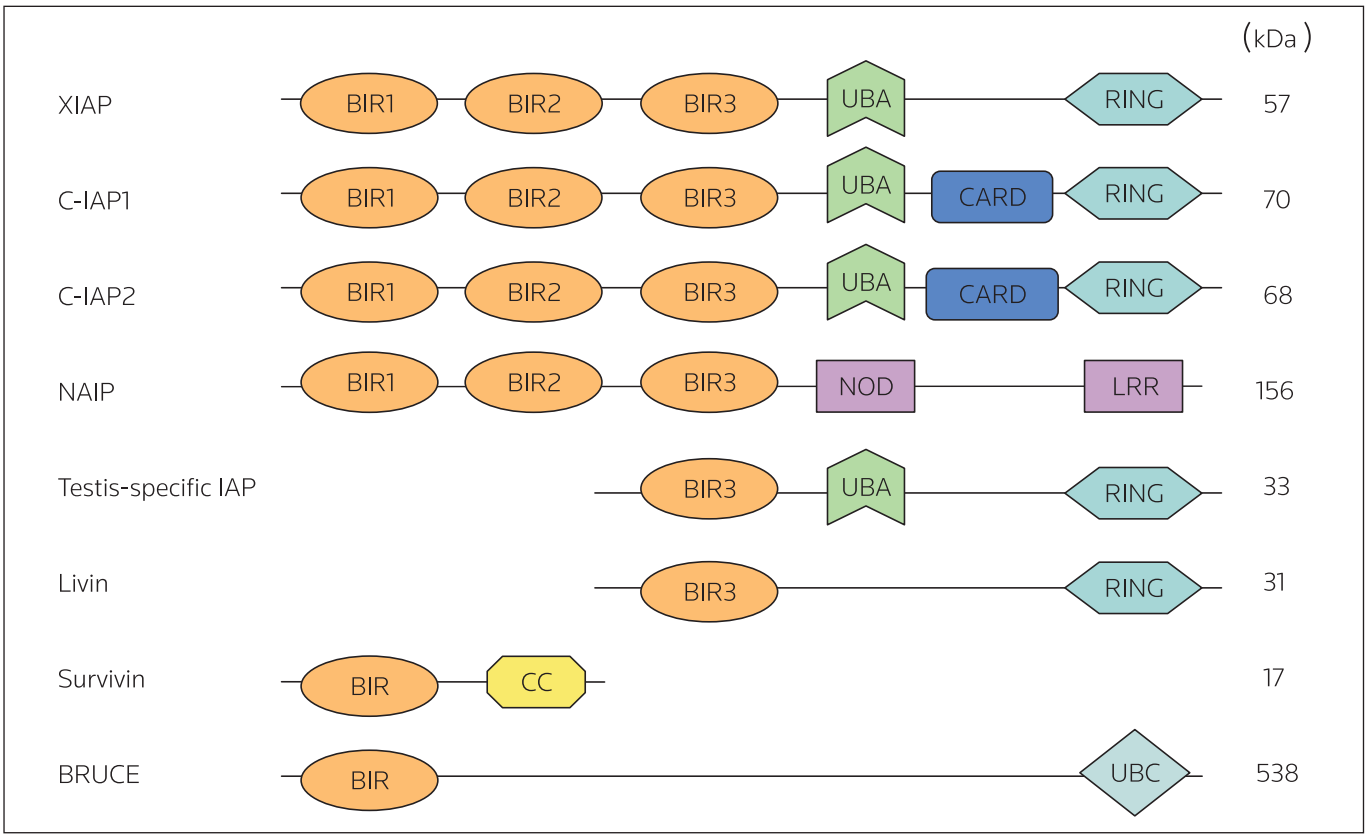


Figure 1. Schematic representation of the human family of inhibitor of apoptosis proteins (IAPs) highlighting key structural domains. For further information on the function of each domain, refer to the text.

-7 (25, 26), recent studies have clarified that caspase-3 is inhibited exclusively by a linker region between BIR1 and BIR2, whereas caspase-7 is inhibited by the same region but also requires the presence of BIR2 for stabilization of the interaction (13). To date, no direct caspase-inhibitory activity has been attributed to BIR1, but rather this domain appears to be a target for phosphorylation and to participate in protein/protein transactions with the tumor necrosis factor (TNF) receptor-associated factor (TRAF) and nuclear factor NF-kappa-B (NF-κB) signaling pathways. It therefore perhaps plays more of a role in modulating the extrinsic death receptor ligand apoptosis pathway (14).

Most IAPs also contain a RING (really interesting new gene) zinc finger domain, which possesses E3 ubiquitin ligase activity, catalyzing both auto- and transubiquitination reactions (28, 29) (Fig. 1). BRUCE, the largest of the IAPs, although lacking a RING, contains a ubiquitin-conjugating domain (UBC) capable of performing a similar function. Post-translational modification of proteins with ubiquitin can result in either proteosomal degradation or stimulate signal transduction. While IAPs promote the proteolytic degradation of caspase-3 and -7, they also advance their own degradation by the proteosome, possibly as a homeostatic control mechanism (29). Thus, the precise contribution of ubiquitination to the overall activity of IAPs remains unclear (14).

Unique to C-IAP1 and C-IAP2 is the caspase activation/recruitment domain (CARD), although the specific function of CARD in IAPs awaits elucidation (13). In addition to three BIR domains, NAIP contains a series of leucine-rich repeat (LRR) sequences together with a nucleotide-binding oligomerization domain (NOD) (Fig. 1). The NOD and LRR domains regulate the BIR interaction with caspase-9 by a unique mechanism requiring the presence of ATP or the absence of the LRR domain (30). The LRR is believed to act as a sensor to trigger NOD-mediated oligomerization (31). Survivin is distinguished by a coiled-coil (CC) domain, required for its interaction with the chromosomal passenger proteins INCENP and borealin, and for its intracellular residency in the nucleus, consistent with an essential role in cell division (32, 33).

Only very recently has a new structural feature been unveiled in the IAPs, an evolutionarily conserved ubiquitin-associated domain (UBA) of the type normally present in proteins known as ubiquitin receptors (34, 35) (see Fig. 1). The role of this domain is still a matter of intense speculation (36). One theory has the domain modulating the C-IAP1-mediated activation of the NF-κB survival signaling pathway, as well as resistance to TNF-α-induced apoptosis (34). Additionally, it is claimed to have a regulatory role in the rapid turnover of autoubiquitinated C-IAP1 and C-IAP2 (35).

XIAP as a target in anticancer drug design

In explaining the ability of IAPs to prevent cells from dying by apoptosis, a number of alternate mechanisms are possible, including: enzyme inhibition, protein/protein interactions, ubiquitination and signal transduction modulation (for review see 13). Different mecha-

nisms have been ascribed to individual IAPs and the degree to which there is crosstalk, independence or built in redundancy in the system is still under question (13, 14, 37). For example, only XIAP is believed to directly inhibit caspase activity (36).

Of all the IAPs described to date, XIAP remains the best characterized and the most potent (38). It has variously been described as the guardian angel (39) and the "Achilles heel" of cancer cells (40). XIAP mRNA is ubiquitously expressed in all adult and fetal tissues in comparison to other IAPs, such as survivin, livin, testis-specific IAP and NAIP. The stability of the protein compared to other IAPs and apparent resistance to ubiquitination-mediated proteosomal destruction (41) have led to speculation that XIAP provides the primary housekeeping function in the prevention of apoptosis by controlling the apoptotic threshold (13). Additional mechanisms that stabilize XIAP include phosphorylation of the BIR1 domain by Akt/PKB and interaction with survivin (42, 43). XIAP is also able to activate NF-κB signaling via an interaction with TAK1-binding protein 1 (44).

XIAP overexpression results in blockade of apoptosis arising from a number of different triggers, including cytotoxic drugs, ionizing radiation and growth factor deprivation (45, 46). The expression of XIAP is frequently elevated in many cancer cell lines, including the NCI-60 panel (18, 47, 48). Significantly, in clinical investigations, XIAP was demonstrated to be overexpressed in a number of different human tumor types (49-51) and high expression is often associated with poor outcome and resistance to chemotherapy (52). Conversely, downregulation of XIAP leads to the induction of apoptosis and sensitization of tumor cells to cytotoxic drugs and ionizing radiation, both in vitro and in vivo (53, 54). However, the removal of XIAP in knockout mice has little detrimental effect on the development of normal tissue (55). Over the last five years, much evidence has accrued clarifying the role of IAPs, and in particular XIAP, in cancer, and these studies clearly point in the direction of XIAP as a potentially validated target in cancer for therapeutic intervention (31, 56-60).

Therapeutic approaches to XIAP inhibition

Two broad-based discovery approaches have been adopted to neutralize the action of XIAP in an attempt to develop potential candidate drugs for eventual clinical evaluation as cancer therapies (58). The first focuses on antisense ASOs and small interfering RNA (siRNA) to knock down or completely ablate the expression of the protein, while the second utilizes a mechanism-based approach to identify small molecules that inhibit or eliminate specific functionality of the protein (see Fig. 2). In terms of ASO, this area will be covered in more detail below. In brief, in addition to AEG-35156, other candidates have emerged, although these are mainly restricted to in vitro applications and proof-of-mechanism studies. A phosphorodiamidate morpholino (AVI-PMO) antisense that targets the ATG start codon in the *XIAP* gene induced apoptosis in human prostate cancer DU 145 cells in vitro, accompanied by an increase in caspase-3 levels (61). A second-generation antisense, ISIS-102369, which targets a region of the *XIAP* gene just upstream from AVI-PMO, also downregulated XIAP, activated caspases and induced cell death, as well as sensitizing human promyelocytic leukemia HL-60 cells to the cytotoxic drug Ara-C (cytarabine) (62). Stable transfection of the human breast cancer cell line MDA-MB-231 with siRNA resulted in 85% knockdown in XIAP mRNA and markedly sensitized these cells to killing by the TNF-related apoptosis-inducing ligand TRAIL (54). Here, increased cell termination correlated with enhanced caspase

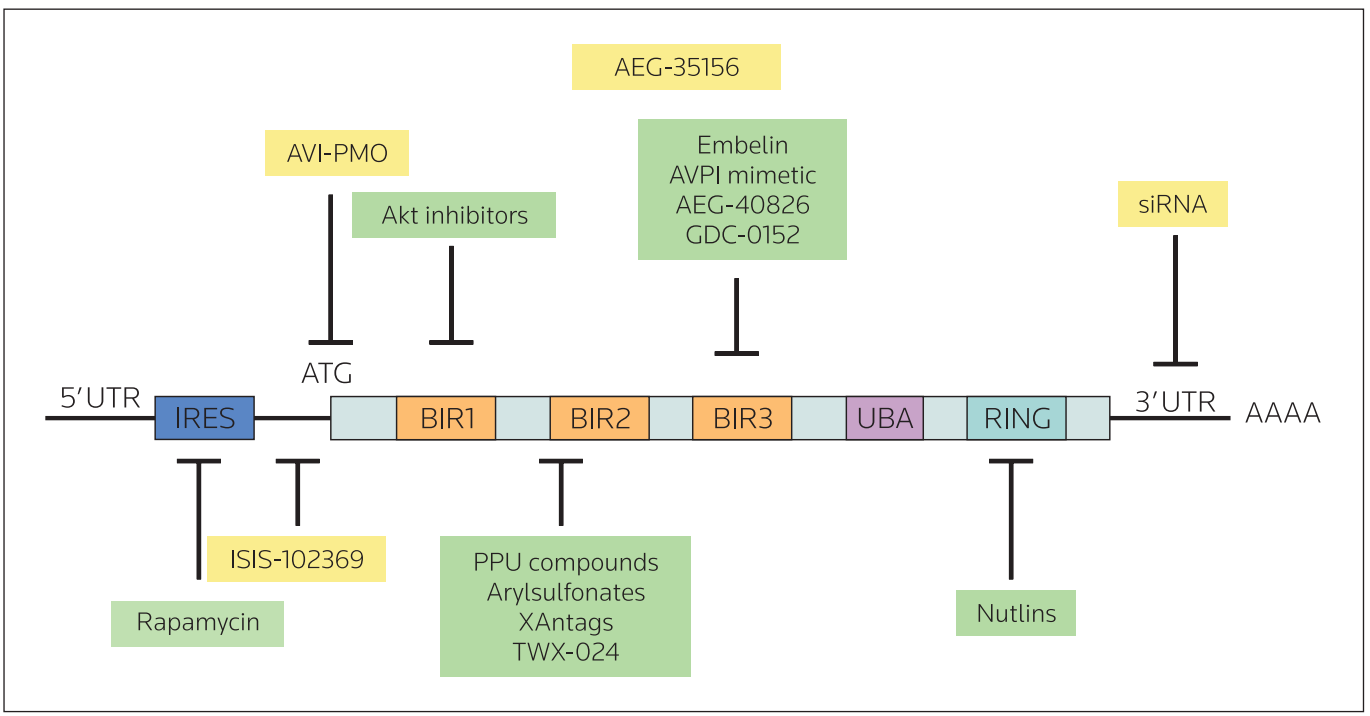


Figure 2. XIAP as a target for anticancer drug design. To date, several classes of compounds have been identified that target different domains and functionality of the protein.

cleavage of the initiator caspase-8, which occupies a central role in the extrinsic death receptor-mediated pathway of apoptosis. Verification that XIAP protein expression can also be downregulated at the gene level by small molecules was reported in multiple myeloma cells using rapamycin, which targets the eponymous mammalian target for rapamycin (mTOR) (63).

Small-molecule inhibitors can be categorized into at least three different classes: 1) destabilizers (targeting BIR1 and RING domains); 2) disruptors of caspase-3 binding (targeting the BIR1-2 linker); and 3) disruptors of caspase-9 binding (targeting BIR3) (14). Examples of compounds that destabilize XIAP are the Akt inhibitors and antagonists of the E3 ubiquitin ligase RING domain of XIAP (e.g., the Nutlins) (64, 65). It is unclear whether altering the balance between autoubiquitination of XIAP and transubiquitination of caspases and their subsequent proteosomal processing would result in therapeutically beneficial net effects (14).

The most spectacular progress has been made recently with agents targeting the caspase binding sites in the BIR1-BIR2 linker and BIR3 domain of XIAP (66, 67). A number of active polyphenylurea (PPU) compounds were identified from a high-throughput screen of mixture-based combinatorial chemical libraries using an in vitro caspase-3 fluorescent derepression assay (68, 69). These are now referred to as XAntags and lead compounds 1396-11 and 1396-12 are progressing through preclinical development (70). Also, using a high-throughput biochemical screen and a combinatorial chemical library has led to the discovery of a novel nonpeptidic arylsulfonamide with the ability to potently disrupt the XIAP/caspase-3 interaction (71). This compound, code-named TWX-024, is presently under preclinical development by Novartis (67).

The discovery that Smac/Diablo acts as an effective endogenous antagonist of XIAP has opened the door to large-scale activity to identify small molecules that mimic (SMCs) the action of this protein (72), resulting in the emergence of at least two new clinical candidates: AEG-40826 from Aegera Therapeutics and GDC-0152 from Genentech (14). Solution of the crystal structure of the XIAP/Smac complex revealed that the four NH_2-terminal amino acid residues of Smac, AVPI (the IAP binding motif, IBM), were sufficient to bind a conserved groove on XIAP BIR3, a site that is also important for caspase-9 inhibition (73). In one of the first proof-of-principle studies, a series of both natural and semisynthetic peptides were designed around this IAP-binding motif sequence (AVPI mimetics) and were capable of displacing caspase-9 from XIAP and inducing apoptosis (74). On the basis of the NMR structure of a Smac peptide complexed with the BIR3 domain of XIAP, a novel series of antagonists was discovered (75). The most potent compounds in this group had nanomolar affinities and promoted cell death in several human cancer cell lines. Significantly, in vivo growth inhibition was recorded in the MDA-MB-231 breast cancer xenograft model in mice. In yet another discovery program, computational structure-based screening of an in-house traditional herbal medicine database was conducted, followed by biochemical testing of selected candidate compounds, resulting in the discovery of embelin from the Japanese *Ardisia* herb (76).

Due to their limited cell permeability, synthetic Smac peptides can be inefficient when tested in cultured cells, limiting their use as potential chemical tools or drug candidates (77). Thus, newer analogues and therapeutic approaches have focused on improving bioavailability, as well as potency (77-79). Bivalent Smac mimetics are capable of ligating two different IBMs, either within a single IAP molecule by intramolecular bridging or between two IAP molecules in an intermolecular configuration. The resulting inhibitors were 100-1,000 times more potent at inducing apoptosis in tumor cells than their monovalent counterparts (80). Others have utilized general structure- and NMR-based approaches to develop nonpeptide or peptidomimetic, small-molecule Smac mimetics with improved cellular permeability (77).

Interestingly, a number of reports have demonstrated quite unexpectedly that SMCs appear to kill cells due to C-IAP1 and C-IAP2 degradation rather than by XIAP antagonism (66, 81-83). Here, it is envisaged that the SMC induces autoubiquitination and degradation of C-IAP1 and C-IAP2, which in turn relieves the repression on ligand-independent, classical and alternative NF-κB signaling. Consequently, NF-κB pathways are activated, which leads to the production of TNF-α. The newly synthesized TNF is subsequently secreted to activate TNF receptors through autocrine or paracrine pathways, and finally apoptosis is activated via the caspase-8-dependent extrinsic pathway.

Antisense as a therapeutic modality in cancer therapy

ASOs are typically 18-25 bases in length and consist of a sequence that is complementary to the messenger (m)RNA of the gene product of interest. It is a therapeutic strategy designed to interfere with and block the transcription and translation of a gene into a protein (see Fig. 3) (84). Specificity is achieved based on the probability that a sequence longer than a minimal number of nucleotides – 13 for RNA and 17 for DNA – occurs only once within the human genome (85). Although recognized for over 25 years that ASOs inhibit gene expression, the exact mechanisms of action are still being elucidated. These include interfering with transcription (1), splicing and maturation of RNA (2), activating RNAase H-mediated RNA cleavage (3), inhibiting nuclear export (4), preventing the initiation of translation (5) and constraining the movement of ribosomes (6) (Fig. 3) (86). Of all these mechanisms, RNAase H-mediated cleavage is believed to be the most critical and is activated by the presence of DNA/RNA hybrids (87). Upon recognition of the hybrid, RNase H degrades the RNA strand, effectively inhibiting the message and preventing new protein synthesis, leaving the antisense strand intact and available to interact with additional RNA molecules by Watson-Crick base pairing hybridization.

As a therapeutic modality in cancer, hundreds, perhaps thousands, of patients have now received antisense treatment, often without major side effects and with clear evidence of target knockdown (85, 88, 89). Several ASOs designed to treat cancer targets have progressed to advanced clinical trials (90). The most intensively studied of these from which the most lessons can be drawn is the 18-mer oligonucleotide targeting the antiapoptotic protein Bcl-2, termed oblimersen (91). Encouraging results were reported in the first clinical trials of oblimersen in non-Hodgkin's lymphoma (92, 93). The treatment was well tolerated, with thrombocytopenia as the dose-limiting toxicity. Pharmacodynamic evaluation of Bcl-2 protein levels revealed that a reduction was achieved in 7 of 16 assessable patients. In a subsequent phase I/II trial in patients with advanced

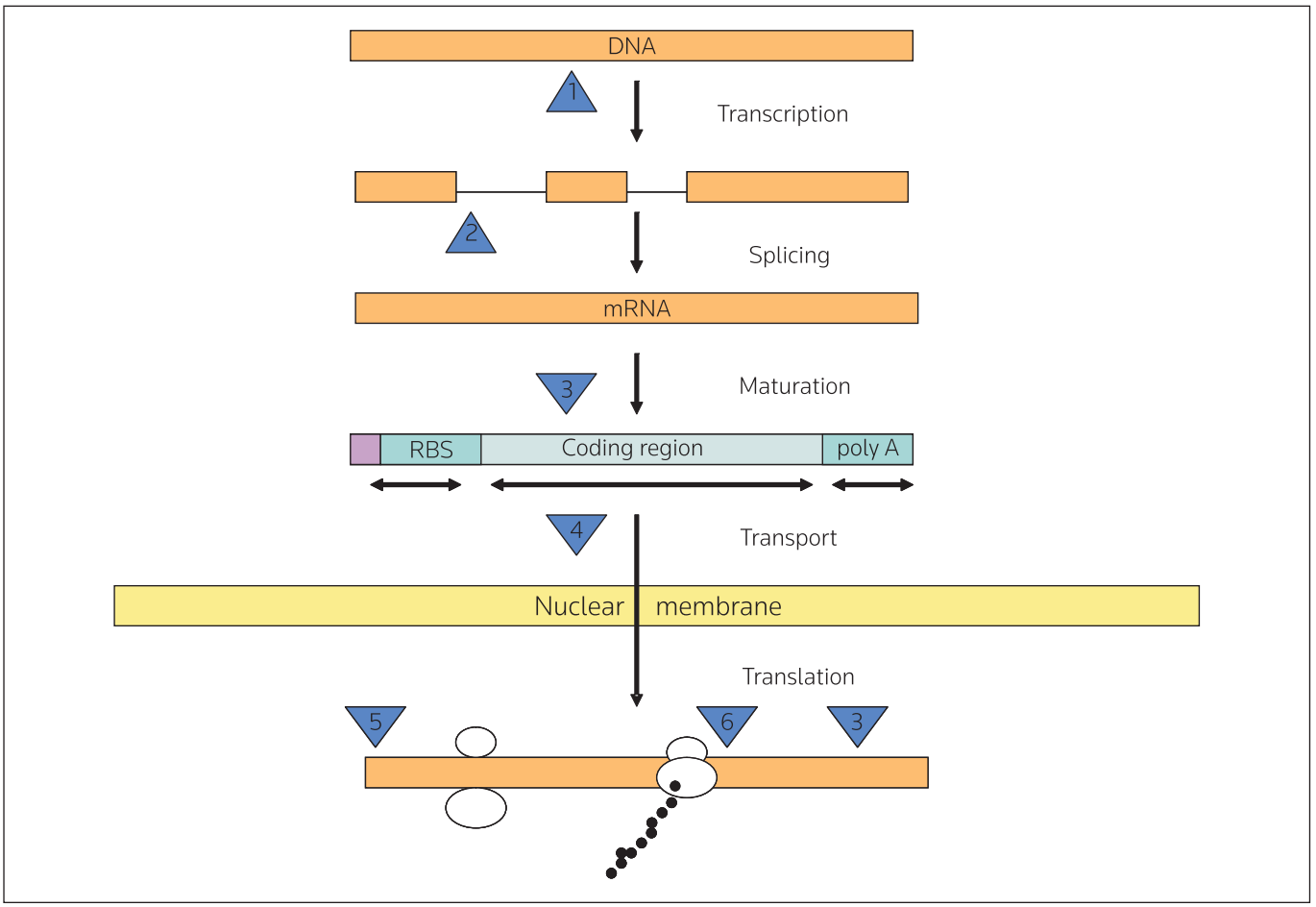


Figure 3. Proposed mechanisms of action of antisense oligonucleotides (ASOs). ASOs can potentially interfere with each stage in the process of DNA transcription, mRNA maturation and nuclear transport and RNA translation (as indicated by the small triangles). RBS, ribosomal binding site.

melanoma, oblimersen was combined safely with full-dose DTIC (dacarbazine) and caused downregulation of Bcl-2 protein in serial biopsy samples, associated with major clinical responses (94). The biological efficacy of oblimersen was also confirmed in other disease states in the phase II context in combination with chemotherapy (95-97). More recently, the results of the first phase III trials were reported in combination with chemotherapy in patients with relapsed or refractory chronic lymphocytic leukemia (98). The ASO was associated with a fourfold increase in response rate and a significant survival benefit was recorded in patients who remained sensitive to chemotherapy.

While encouraging, the results from these clinical trials were not sufficient to support its regulatory acceptance either by the FDA or the EMEA (99). In the original submission to the FDA, Genta presented data on 771 patients. Evidence showed that the combination of oblimersen and dacarbazine significantly increased progression-free survival (PFS) over dacarbazine alone. However, no concomitant increase was observed in overall survival – the primary endpoint – and as a consequence, the submission for approval was rejected on the basis of a lack of clinical activity (100). While survival benefits were demonstrated in the phase III setting, regulatory approval still remains elusive (101).

Other antisense molecules have also reached later stages of clinical evaluation but have perhaps fared even less well than oblimersen. ISIS-3521, which targets the key signal transduction kinase PKC-α, was investigated exhaustively in phase II and III trials in non-small lung cancer (NSCLC). Nonetheless, the drug was discontinued when the results of more extensive phase III trials revealed that the antisense component provided no real improvement in response or survival benefit (102, 103). Other antisense targets under later-stage clinical evaluation include: H-Ras (104), Raf kinase (105), clusterin (106) and survivin (107). Unfortunately, taken together, these trials provide little further support for significant clinical activity.

The majority of the antisense molecules utilized in the clinical trials reported above were synthesized by replacing the phosphodiester backbone of the nucleotide with a phosphorothioate (PS) linkage (85). Termed first generation, this chemical substitution increases resistance to nuclease degradation and extends half-life significantly (108, 109) (Fig. 4). However, the failure of these first-generation molecules to produce more spectacular antitumor activity was also blamed on the presence of polyanionic sulfur, resulting in inadequate penetration into tumor tissue and variable knockdown of the target (110, 111). In addition, many of the side effects observed in patients receiving PS antisense, such as stimulation of immune reactions,

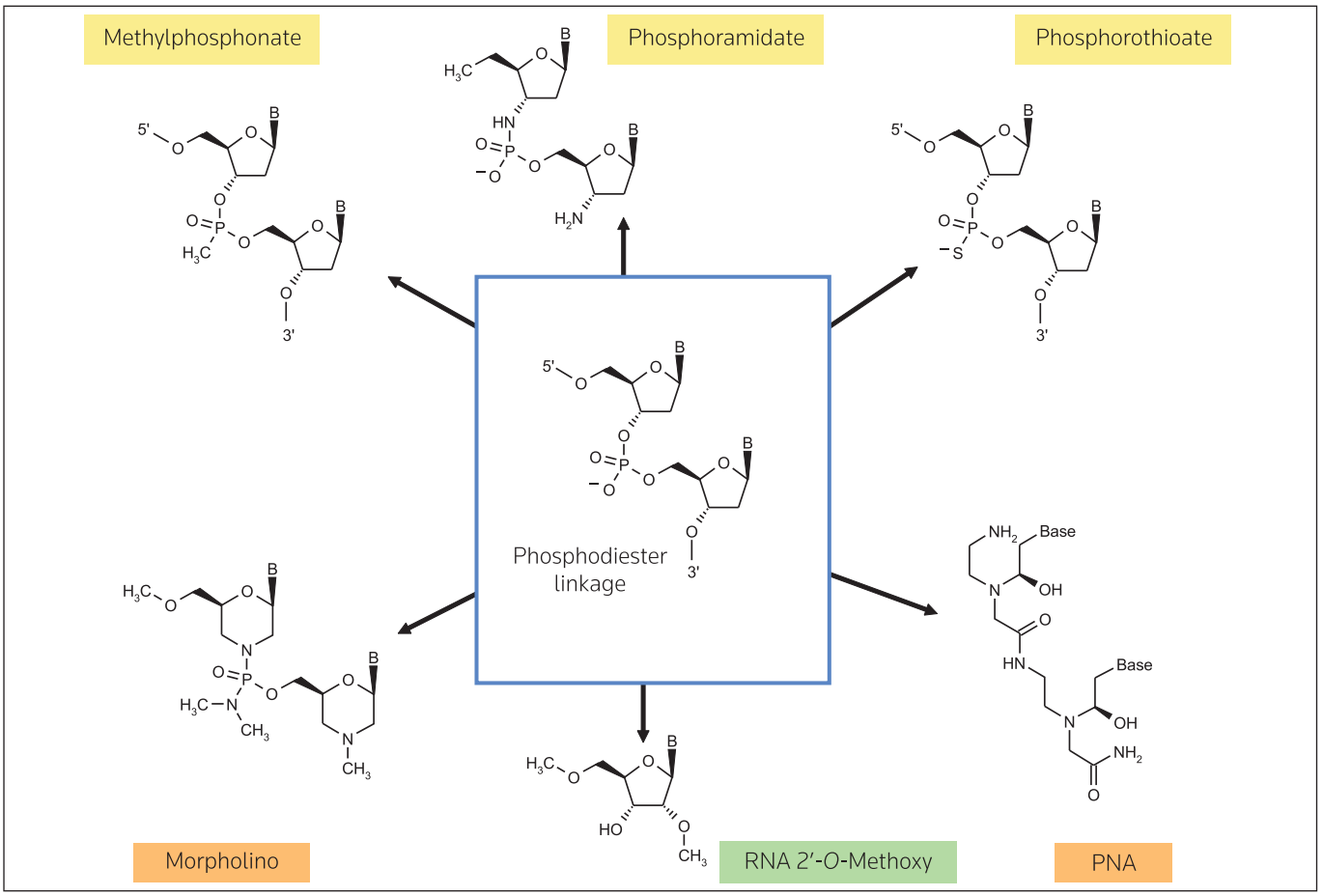


Figure 4. Typical examples of chemical modifications incorporated into the backbone of antisense oligonucleotides: yellow, first-generation chemistry; green, second-generation chemistry; and brown, third-generation chemistry.

extensive binding to thrombin and a decrease in coagulation, are believed to be due to off-target effects induced by polyanionic sulfur and specific sequences such as CpG and polyG motifs (112).

Two approaches have been embraced to improve tissue/tumor penetration and reduce off-target effects. The first entails packaging antisense into a number of different drug delivery systems, including liposomes and nanoparticles (for reviews see 89, 113, 114). The second utilizes chemical modifications to the backbone, resulting in the development of first-, second- and third-generation chemistries (see Fig. 4). Substitution of an oxygen with aminoalkyl or methyl yields phosphoroamidate and methylphosphonate. Substitutions at the 2′-position with *O*-methyl and *O*-methoxyl produce antisense with reduced toxicity, but these are also unable to activate RNase H and probably work by interfering with translation (115). Mixed-backbone second-generation chemistry consists of stretches of RNA and DNA incorporating both PS and 2′-*O*-methyl modifications (116). These alterations prevent degradation by nucleases but still contain a region of DNA allowing for efficient RNase H activation and target knockdown. Third-generation chemistry is characterized by more dramatic structural changes and includes morpholino (117) and peptide nucleic acids (PNAs) (118-120). PNAs represent a new mechanistic category that can bind to genomic DNA and RNA to form a triple helical structure. PNAs are highly resistant to enzymatic cleavage and may represent the most stable antisense molecules within cells (119).

Design of AEG-35156

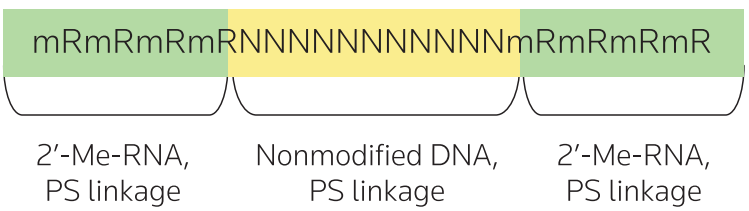
In 2002, Aegera Therapeutics, which owned intellectual property rights to the *XIAP* gene (http://www.aegera.com/intellectual_property.php), and Hybridon (Cambridge, USA; now merged into Idera Pharmaceuticals) announced a collaboration to develop an antisense therapy targeting XIAP (121). Initially, a library of 96-plus nonoverlapping 19-mer mixed-backbone oligonucleotides (MBOs) (covering the majority of the 2540-bp cDNA of the *XIAP* gene) was synthesized, composed of two 2′-*O*-methyl RNA residues flanking either end and a central core of 15 phosphodiester DNA residues (53). Several of the antisense candidates (including G4 and C5) were shown to knock down XIAP mRNA by 50-70% in human NSCLC NCI-H460 cells, and the G4 analogue also effectively decreased protein expression by 60%. Treatment of NCI-H460 cells with G4 alone induced cell death, accompanied by degradation of procaspase-3 and poly(ADP)ribose polymerase (PARP) and nuclear DNA condensation and fragmentation, consistent with apoptosis, and synergized with a number of cytotoxic drugs, including doxorubicin and etoposide (53). Significantly, the G4 analogue produced dose-dependent

antitumor activity in vivo against NCI-H460 xenografts, concomitant with an 85% decrease in XIAP protein expression in the tumor. Moreover, the histology of the xenografts treated with G4 indicated that the growth inhibition was associated with efficient tumor cell death. These initial studies provided proof of mechanism that ASOs could effectively knock down XIAP protein and proof of concept that XIAP knockdown could result in antitumor activity and sensitize tumor cells to cytotoxic drugs. In a separate study, the G4 analogue also enhanced the activity of radiotherapy (122).

The first preliminary reports on AEG-35156/GEM-640 appeared in 2004 (123, 124), where the ASO was synthesized using β-cyanoethylphosphoramidate chemistry (116). AEG-35156 is a 19-mer MBO, but is fully phosphorothioated, consisting of an 11-nucleotide DNA core flanked at the 3′ and 5′ ends by four 2′-*O*-methyl RNA residues (see Fig. 5). The design features of the antisense include the use of second-generation chemistry for increased stability, greater potency, preservation of RNase H activation, reduced toxicity, elimination of off-target effects and the potential for oral formulation (57). AEG-35156 spans an exon–intron boundary of XIAP mRNA; it does not contain CpG motifs and showed little evidence of immunostimulation in a mouse splenomegaly assay.

PRECLINICAL PHARMACOLOGY

The ability of AEG-35156 to knock down XIAP mRNA and protein was initially evaluated in a panel of human cancer cell lines, including NCI-H460, PANC-1 pancreatic cancer, cisplatin-resistant A2780 ovarian cancer, MDA-MB-231 breast cancer and PC-3 prostate cancer cells (125, 126). The ASO was highly potent at reducing XIAP mRNA, with an EC_{50} concentration ranging from 8 to 32 nM, and achieved knockdown of protein in excess of 80%. These results represent a substantial improvement over the first series of antisense molecules studied, which were normally only effective at micromolar concentrations (53). The enhanced activity was ascribed to the increased stability of second-generation chemistry and optimization of sequence selectivity (126). Furthermore, AEG-35156 increased caspase-mediated cleavage of the DNA repair protein PARP and sensitized PANC-1 and NCI-H460 cells to apoptosis via the extrinsic pathway. Loss of mRNA was demonstrated to involve RNase H. Significantly, AEG-35156 appeared to specifically target XIAP and did not decrease the expression of other closely related members of the IAP family, such as C-IAP1 (126). Repeated transfection of cells with AEG-35156 resulted in a more profound knockdown of the target, suggesting that in addition to RNase H activation, AEG-35156-mediated loss of XIAP protein may also result from translational interference or altered splicing (see Fig. 3) (126). AEG-35156 also substantially decreased XIAP protein expression in a panel of human ovarian cancer cell lines concomitant with cleavage of PARP, activation of caspase-3 and a reduction in cell number (127). The presence of mutant p53 in the cisplatin-resistant A2780 cell line had no major impact on the ability of AEG-35156 to induce apoptosis.



Property	Description
Length	19 nucleotides
Structure	4 · 4 MBO; a chimeric oligonucleotide with four 2′-*O*-methylribonucleotide residues at both the 3′ and 5′ ends, flanking a core of 11 2′-deoxyribonucleotide residues
Backbone	Fully phosphorothioated
MW (approximate)	< 6200 Da for free acid, < 6600 Da for sodium salt
CpG motifs	None
Target region	Coding region of XIAP (gene symbol = *BIRC4*), spanning an exon–intron boundary
Immunostimulation	Not immunostimulatory in a mouse splenomegaly assay

Figure 5. Drug design features of AEG-35156.

Table I. *The antitumor activity of AEG-35156 against a panel of human xenografts administered as either a single agent or in conjunction with cytotoxic drugs.*

Human xenograft	Lineage	Dose (mg/kg)	Schedule	Cytotoxic*	Efficacy# (%)	Target KO	Ref.
Colon	LS 174T	10	5 d on, 2 d off, 3 cycles	None	30	ND	126
Colon	LS 174T	25	5 d on, 2 d off, 3 cycles	None	60	ND	126
Lung	NCI-H460	25	5 d on, 2 d off, 3 cycles	None	40	Yes	126
Lung	NCI-H460	10	5 d on, 2 d off, 3 cycles	Docetaxel	56	Yes	126
Lung	NCI-H460	25	5 d on, 2 d off, 3 cycles	Docetaxel	80	Yes	126
Lung	NCI-H460	25	5 d on, 2 d off, 3 cycles	Cisplatin	40	Yes	126
Prostate	PC-3	10	5 d on, 2 d off, 6 cycles	None	40	ND	126
Prostate	PC-3	25	5 d on, 2 d off, 6 cycles	None	80	ND	126
Prostate	PC-3	10	5 d on, 2 d off, 6 cycles	Docetaxel	90	ND	126
Prostate	PC-3	25	5 d on, 2 d off, 6 cycles	Docetaxel	100	ND	126
Prostate	PC-3	25	5 d on, 2 d off, 6 cycles	Carboplatin	80	ND	126
Ovarian	Cisplatin-resistant A2780	10	5 d on, 2 d off, 6 cycles	None	30 d	Yes	127
Ovarian	Cisplatin-resistant A2780	25	5 d on, 2 d off, 6 cycles	None	16 d	Yes	127
Breast	MDA-MB-231	25	5 d on, 2 d off, 9 cycles	None	80	ND	126

*Docetaxel was administered at a dose of 30 mg/kg on days 11 and 18 in NCI-H460 and at 15 mg/kg on days 4 and 11 in PC-3. Cisplatin was administered at 6 mg/kg on days 11 and 18 and carboplatin at 120 mg/kg on day 4. #Efficacy is reported as % reduction in tumor volume in drug-treated compared to control (T/C), except in cisplatin-resistant A2780, where activity is reported as an increase in survival in days.

Preclinical verification of proof of mechanism was obtained in primates, where continuous i.v. infusion to cynomolgus monkeys reduced XIAP protein levels in liver and kidney in a dose-dependent manner by between 60% and 80%, corresponding to steady-state plasma levels of 0.250 and 1.25 µM, respectively (128).

The antitumor activity of AEG-35156 was studied extensively by Aegera scientists against a panel of five different human xenografts representing a broad spectrum of different disease types (Table I) (57, 123, 126, 127). Here, the ASO was administrated by i.p. injection in repeating cycles of 5 days on and 2 days off, either as a single agent or in combination with conventional cytotoxic drugs. As a single agent, dose-dependent growth-inhibitory activity of up to 80% was observed (126). A significant increase in survival was also recorded in mice bearing cisplatin-resistant A2780 ovarian cancer xenografts, although this effect was more pronounced at 10 mg/kg compared to 25 mg/kg (127). When assessed, antitumor activity was associated with XIAP protein knockdown in xenografts (126) or with a significant reduction in the number of viable cells present (127). AEG-35156 was relatively inactive as a single agent in the NSCLC NCI-H460 xenograft model, but when combined with a suboptimal dose of docetaxel (30 mg/kg on days 11 and 18) a dramatic increase in antitumor activity was noted. However, this effect was not replicated by a combination of the ASO and cisplatin (6 mg/kg on days 11 and 18) in this model. Nonetheless, combining the antisense with another platinum agent (carboplatin, 120 mg/kg on day 4) did result in synergy in the PC-3 xenograft model, while the combination with docetaxel (15 mg/kg administered on day 4 and 11) achieved complete tumor regression that was sustained over 98 days. Antitumor activity was accompanied by few overt signs of toxicity or a reduction in body weight, and a series of oligonucleotide controls, including AEG-35191 (4-base mismatch), AEG-35185 (nonsense) and AEG-35187 (scrambled), were without activity both as single agents and in combination with cytotoxic drugs. Taken together, these data revealed that AEG-35156 had promising antitumor activity as a single agent in several xenograft models and also combined synergistically with (certain) clinically relevant cytotoxic drugs.

PHARMACOKINETICS AND METABOLISM

Preclinical pharmacokinetic studies were conducted in cynomolgus monkeys after administration of AEG-35156 as a 7-day continuous infusion over a wide range of doses (57, 124, 128). The ASO exhibited linear, dose-dependent pharmacokinetics, with steady-state levels normally achieved after 6 days (128). In keeping with most PS ASOs, after the end of the infusion plasma levels fell rapidly, with a terminal elimination half-life of 1 h. Metabolites of AEG-35156 were also detected in plasma and tissues and identified as predominantly the N-1 and N-2 products (lacking one or two nucleotides). These metabolites could account for between 30% and 50% of the total material by the end of the treatment period and are anticipated to retain biological activity (125).

SAFETY

AEG-35156 was produced to GMP-grade material to undergo formal preclinical toxicology and pharmacokinetic evaluation to GLP standards in rodents and primates (57). Toxicology involved three different genotoxicity assays, a safety study in cynomolgus monkeys to evaluate cardiac, respiratory and neurological toxicity, and range-finding studies in rats. The cynomolgus monkey was adopted as the most appropriate animal species in which to derive the starting dose for man. In the rat studies, AEG-35156 was administered by bolus i.v. injection, while in primates it was administered by continuous

infusion to mirror the proposed phase I trial schedule involving two 7-day cycles with a 14-day postinfusion rest period between treatments and a 14-day observation period post-treatment. In all three genotoxicity tests AEG-35156 proved negative, and in cardiovascular, respiratory or neurological evaluations no treatment-related effects were observed, with the exception of a slight activation of the alternate complement pathway, which was related to the peak plasma concentration. It was concluded that the adverse events observed in rats and monkeys were consistent with the well-characterized side effects caused by first- and second-generation phosphorothioated oligonucleotides and were due mainly to accumulation of AEG-35156 and metabolites in the liver and kidney (57). Evidence was also presented of limited immunostimulation; however, since AEG-35156 lacks CpG motifs, the mechanism of action remains unclear (57).

CLINICAL STUDIES

The first phase I clinical trial of AEG-35156 commenced in March 2004 in the U.K. at clinical centers in Manchester and Edinburgh. Based on preclinical toxicology, the starting dose was selected at 48 mg/m^2/day, which was one-tenth of the maximum tolerated dose (MTD) in cynomolgus monkeys. AEG-35156 was administered originally as a 7-day infusion as part of a 21-day treatment cycle, but the trial protocol was subsequently amended to include a 3-day continuous infusion (129, 130). A total of 38 patients were entered into the trial in 7 cohorts and the drug was generally well tolerated, with most adverse events manifesting either as grade 1 or 2 in severity or asymptomatic (131). The MTD was 125 mg/m^2/day for the 7-day regimen and ≤ 213 mg/m^2/day for the 3-day regimen, with dose-limiting toxicity comprising elevated hepatic enzymes (in particular ALT and AST, and less frequently GGT), hypophosphatemia and thrombocytopenia. Within the patient group, 7 subjects had stable disease, 23 had progressive disease and 8 were not assessable. Two partial responses were observed, one in a breast cancer patient who received 125 mg/m^2/day (3-day infusion) and the other in a malignant melanoma patient who received 213 mg/m^2/day (7-day infusion), but subsequent scans revealed the development of new lesions in both. Four patients also had mixed responses, with certain areas of tumor apparently responding to treatment while other areas of tumor continued to progress. Of note was the observation of a dramatic clearance in circulating tumor lymphocytes in an individual with non-Hodgkin's lymphoma, which corresponded to a clear reduction in XIAP mRNA expression in peripheral blood mononuclear cells (PBMCs) (131). Pharmacodynamic and biomarker studies were an integral component of this trial and are presented below.

The pharmacokinetics of AEG-35156 were evaluated utilizing a capillary electrophoresis plasma assay validated to GLP standards (124). AEG-36156 exhibited linear kinetics, with no evidence of saturation of clearance mechanisms even at the highest doses studied (131). Steady-state plasma concentrations were evident after 24 h and ranged from (approximately) 0.4 to 4.5 µg/mL after administration of 48-213 mg/m^2/day. After discontinuation of the infusion, mean plasma concentrations fell precipitously with a half-life of 1 h. Population pharmacokinetic modeling established that the plasma concentration–time curves for the elimination phase were best described by a biexponential decline and a two-compartment model, where the terminal half-life was 24 h.

In the light of these encouraging clinical data from the first phase I trial (131), a number of other phase I and II trials (eight in total), employing predominantly a 2-h infusion schedule, have been initiated (for additional information: http://www.aegera.com/clinical_trials_aeg35156.php and http://clinicaltrials.gov/ct2/results?term=AEG35156).

The first phase I trial of AEG-35156 administered as a 2-h infusion adopted a schedule of once-weekly administration following three daily loading doses in week 1, and was conducted in Manchester. Thirty adult patients were recruited in total. In a preliminary account of this trial, data were presented on 23 subjects who had completed at least one 3-week treatment cycle at doses ranging from 60 to 500 mg (132). Treatment was generally well tolerated, the most common toxicities being grade 1 chills/pyrexia, rapidly reversible increases in aPTT at the end of infusion and mild transaminitis. Again, pharmacokinetics were dose-dependent and linear from 60 to 500 mg AEG-35156, with a peak plasma concentration (C_{max}) of 55 µg/mL at 500 mg. Decreases in XIAP mRNA were observed in PBMCs at dose levels ≥ 240 mg. One patient with non-Hodgkin's lymphoma had a rapid clearance of circulating tumor cells and reduction in nodal disease after only a single infusion.

In one of the more recent phase I/II trials, XIAP antisense was administered by 2-h infusion combined with chemotherapy in the treatment of refractory/relapsed acute myeloid leukemia (AML) (133, 134). AEG-35156 was well tolerated up to a dose level of 350 mg/m^2 in combination with idarubicin and cytarabine. At the highest dose levels, AEG-35156 reproducibly reduced levels of XIAP mRNA in circulating blasts. The combination of AEG-35156 and the two cytotoxic drugs produced remissions in patients who had not responded to a single induction therapy or were in first relapse after a short initial complete response.

Pharmacodynamic and biomarker studies were treated as a high priority during the clinical evaluation of AEG-35156 and as a consequence the assays utilized were subject to extensive validation (135-139). Two types of assays were employed: proof of principle/mechanism (POM) aimed at measuring knockdown of XIAP mRNA or protein, and proof of concept (POC) to determine whether knockdown of XIAP resulted in tumor cell death. None of these biomarker assays, however, were intended to substitute for the primary endpoints of the trial, but were accorded the status of either secondary or tertiary endpoints (140). The POM assays included Western blot analysis, quantitative RT-PCR (qRT-PCR) and immunohistochemistry (135, 136). The POC assays included two serological cell death ELISAs (M30 and M65) and two different IHC assays for the measurement of cleaved caspase-3 and cleaved PARP in tumor tissue. M30 and M65 are sandwich ELISA assays that determine in either plasma or serum different circulating forms of the epithelial protein cytokeratin-18 (CK-18) (141-143). The M30 ELISA assay utilizes the M5 antibody as a catcher and the M30 antibody to detect CK-18 fragments that contain a neoepitope at positions 387-396 generated by the action of caspase-3, -7 and -9 activated during the early stages of apoptosis (Fig. 6) (144, 145). Thus, the M30 ELISA is proposed as a specific assay for apoptosis (146). M65 also detects cleaved fragments (143), although it uses a different detection antibody from M30 (namely M5) that does not distinguish between the full-length protein and its fragments. Thus, M65 theoretically meas-

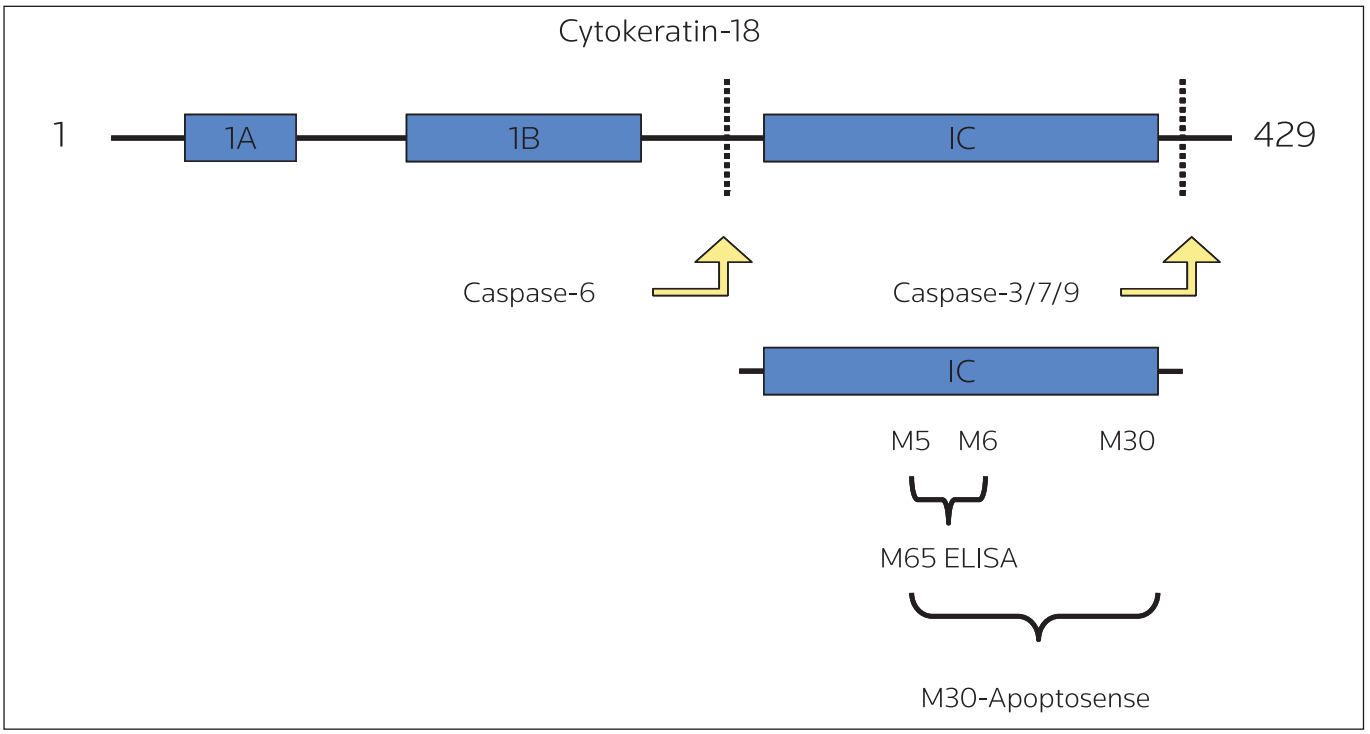


Figure 6. Schematic representation of the cytokeratin-18 (CK-18) epitope map targeted by the M30 and M65 sandwich ELISA assays. In the case of the M65 ELISA the M6 antibody acts as the catcher and M5 as detection antibody. For the M30 ELISA assay M5 is the catcher and M30 the detection antibody. Proposed caspase cleavage sites of CK-18 are also indicated.

ures both caspase cleavage (apoptosis) and cellular release of intact CK-18 (necrosis). Both assays have now been applied extensively in clinical trials as biomarkers of cell death (143, 147-149).

In the first clinical trial of AEG-35156, a dose-dependent decrease in XIAP mRNA was detected in PBMCs by qRT-PCR 72 h after the start of both the 3- and 7-day infusions. Although the magnitude of this reduction could approach 50-60%, across the whole patient population the mean percentage decrease from baseline was 22% for the 7-day infusion and 19% for the 3-day infusion, and the effect was often not sustained. Indeed, occasionally, the phenomenon of rebound was evident, where after initial knockdown mRNA expression levels bounced back to values greater than those prior to treatment. Reductions in XIAP protein levels were also detected by Western blot analysis in PBMCs, but these were more sporadic on the 7-day than the 3-day schedule. Nine paired tumor biopsy samples were obtained in total, but only five pairs were assessable by IHC for XIAP, cleaved caspase-3 and cleaved PARP. No conclusive drug-related changes could be demonstrated between pre- and post-infusion biopsies. Plasma profiles of the CK-18-derived cell death markers —the M30 and M65 antigens— were obtained in 28 patients and these often exhibited dose-dependent elevations of > 100% associated with drug infusion. In most instances, the biomarker peak occurred after 3 days, consistent with induction of cell death corresponding to AEG-35156-mediated knockdown of XIAP. Occasionally, a patient also had an M30/M65 plasma profile signature characterized by a gradual upward trajectory, suggestive of continuing disease progression. One patient who had a nonepithelial CK-18-negative tumor showed marked increases in M65 antigen levels of 300-500%, correlating with the increase in AST observed as a result of drug-related hepatic toxicity (131). A similar profile of M30 and M65 biomarker changes was also observed on the 2-h infusion schedule (150).

Extensive biomarker analysis was also conducted during the more recent phase I/II trials where XIAP antisense was administered by 2-h infusion and combined with chemotherapy in the treatment of refractory/relapsed AML (133). XIAP mRNA levels were determined in CD3/CD19-depleted mononuclear cells by RT-PCR and apoptosis induction in RBC-lysed whole blood was measured by annexin V positivity and/or changes in mitochondrial membrane potential. Dose-dependent decreases (up to 90%) in mRNA levels were evident, while the dose of 350 mg/m^2 was effective in knocking down XIAP mRNA levels in circulating blasts. This was accompanied by the induction of apoptosis preferentially in the CD34$^+$38$^-$ stem cell compartment.

FUTURE PROSPECTS

Anticancer drug discovery is now firmly rooted in the era of rational design, having graduated from whole-scale random screening. Nonetheless, a mechanism-based therapy (MBT), even one exhibiting the exquisite specificity of an antisense oligonucleotide, is only likely to be as good as the molecular target it is directed against. Spectacular failures in targeted therapies have been reported. XIAP is only one member of a family of proteins and the degree of crosstalk, independence or built-in redundancy among these different endogenous inhibitors of apoptosis is still under debate. AEG-

35156 has been demonstrated to be both a potent and selective inhibitor of XIAP in vitro, resulting in a marked downregulation in mRNA, decreased expression of protein and induction of apoptosis. In vivo AEG-35156 exhibits broad-spectrum antitumor activity against a panel of human cancer xenografts and synergizes with conventional cytotoxic drugs. Many of these properties were replicated in subsequent clinical trials: 1) the drug is well tolerated; 2) there is ample evidence in surrogate tissues of suppression of XIAP mRNA and reduction in XIAP protein levels; and 3) induction of apoptosis was evident. There were also indications of clinical responses, especially in lymphoma, as well as encouraging prospects for combining the antisense with conventional chemotherapy. It is likely that AEG-35156 will continue to undergo clinical evaluation for several years to come. Ultimately, the clinical success or failure of AEG-35156 will depend on the degree to which XIAP is solely responsible for maintaining the inhibition of apoptosis in different human cancers.

SOURCES

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