

 CASE HISTORY

## Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease

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**Abstract** | Aptamers are oligonucleotide ligands that are selected for high-affinity binding to molecular targets. Pegaptanib sodium (Macugen; Eyetech Pharmaceuticals/Pfizer) is an RNA aptamer directed against vascular endothelial growth factor (VEGF)-165, the VEGF isoform primarily responsible for pathological ocular neovascularization and vascular permeability. After nearly a decade of preclinical development to optimize and characterize its biological effects, pegaptanib was shown in clinical trials to be effective in treating choroidal neovascularization associated with age-related macular degeneration. Pegaptanib therefore has the notable distinction of being the first aptamer therapeutic approved for use in humans, paving the way for future aptamer applications.

### Age-related macular degeneration

A disease process characterized by deterioration of the macula that results in a loss of sharp central vision; non-exudative ('dry') and exudative ('wet') forms occur.

In December 2004, the US FDA approved pegaptanib sodium (Macugen), an anti-vascular endothelial growth factor (anti-**VEGF**) RNA aptamer, for the treatment of all types of neovascular age-related macular degeneration (AMD). Pegaptanib's approval represents a milestone in drug development in that it is the first aptamer to be successfully developed as a therapeutic agent in humans. Two major lines of research are reflected in this achievement: the development of aptamers as biological agents with applications in therapeutics, diagnostics and research; and the validation of VEGF as a major regulator of aberrant and excessive blood vessel growth and permeability in the eye. Pegaptanib is also the first anti-angiogenic therapy indicated for the treatment of neovascular AMD and one of the two anti-VEGF agents (along with the monoclonal antibody bevacizumab (Avastin; Genentech), an anticancer agent; for a comprehensive review, see REF. 1) approved for human use. In this review, the development of aptamers as therapeutics will be discussed, with a focus on the early advances, preclinical characterization and, ultimately, successful clinical trials of pegaptanib in patients with ocular neovascular disease.

### Aptamers as therapeutic agents

**Aptamers mimic cellular and viral machinery.** Since the advent of genetic engineering and molecular biology in the 1960s and 1970s, it has become commonplace to encounter highly specific interactions between proteins and oligonucleotide sequences; examples include restriction enzymes and their target cleavage sequences, and transcription factors and their sequence-specific DNA

promoter elements. The concept of the modulation of protein function by naturally occurring nucleic acids has emerged more recently, primarily through the study of virus–host interactions. For example, a small viral RNA produced during adenovirus infection binds to and inhibits the function of a cellular kinase, effectively shutting down an arm of the cell's antiviral defences<sup>2</sup>. Another example is the human immunodeficiency virus-1 5' transactivation response (TAR) element. An essential step in viral transcription requires an interaction between TAR, the viral protein Tat and a cellular cyclin (CycT1); when TAR binds to CycT1, it greatly enhances the protein–protein interaction between CycT1 and Tat<sup>3</sup>.

Oligonucleotide–protein interactions, recognized as ubiquitous in the biological world, have now been successfully exploited for the development of an entirely new class of therapeutics. Aptamers (from the Latin *aptus*, to fit, and the Greek *meros*, part or region)<sup>4</sup> are RNA or DNA oligonucleotides that have been selected for their ability to bind proteins with both high affinity and high specificity. The term has also been extended by some to include peptides with protein-binding properties<sup>5,6</sup>, but for the most part and for the purposes of this review the term is used to describe oligonucleotide agents. Currently, one aptamer therapeutic is commercially available and an additional two are in clinical development.

**Selection of aptamers that target proteins.** The selection of aptamers with specificity for virtually any protein target has become straightforward following the advent of systematic evolution of ligands by exponential

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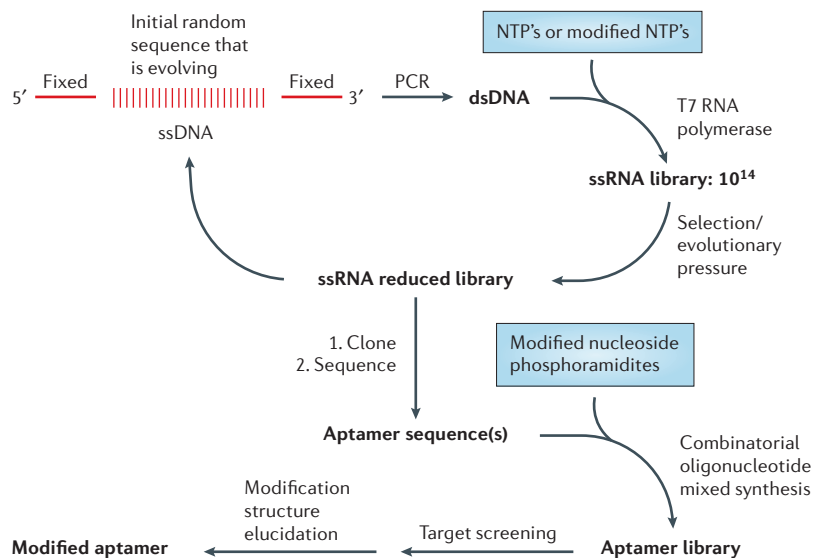


Figure 1 | A depiction of the SELEX process<sup>7</sup>. NTP, nucleotide triphosphate; PCR, polymerase chain reaction; SELEX, systematic evolution of ligands by exponential enrichment.

enrichment (SELEX) (FIG. 1)<sup>7</sup>, a process developed in the laboratory of Larry Gold at the University of Colorado<sup>8</sup>. This process begins with a population of random RNA or DNA oligonucleotides, typically 20–40 nucleotides long. Randomization is achieved simply by using a mixture (usually not equimolar) of the four standard nucleotides during solid-phase synthesis. The randomized oligomer region is flanked by fixed regions containing binding sequences for reverse transcriptase (RT) and polymerase chain reaction (PCR) primers, a promoter sequence for T7 RNA polymerase, and perhaps restriction endonuclease sites for cloning. Whereas in principle a randomized 40-nucleotide library could contain in excess of  $10^{24}$  different sequences, in practice a standard library yields up to  $10^{15}$  different candidate aptamers<sup>9</sup>. Aptamers that bind a target are selected, amplified by RT-PCR and then reselected (at higher stringency, if desired), with the process repeated as needed to achieve the desired affinity and specificity. After cloning and sequencing, selected aptamer sequences then can be synthesized chemically using solid-phase phosphoramidite chemistry.

Unmodified nucleic acids are very susceptible to nuclease attack and are therefore generally unsuitable as therapeutic agents. As examples, an unmodified antisense oligonucleotide had a half-life in serum of less than a minute<sup>10</sup>, whereas an unmodified aptamer against thrombin exhibited an *in vivo* half-life of 108 seconds<sup>11</sup>. Pieken *et al.*<sup>12</sup> demonstrated that substituting the 2' hydroxyl of the ribose moiety of pyrimidines with either fluorine (F) or an amino group (NH<sub>2</sub>) increased the nuclease resistance of ribozymes by several orders of magnitude. For a similar approach to be useful in generating nuclease-resistant aptamers, the substituted nucleotides must be acceptable substrates for both T7 polymerase and RT. Both F- and NH<sub>2</sub>-substituted ribonucleotides fulfil this condition, and they were subsequently used in the development of resistant aptamers

against VEGF<sup>13,14</sup> (see below). A further modification that improves RNA oligonucleotide stability against nucleases is the substitution of 2'-O-methyl (2'-OMe) at the 2'-hydroxyl position of purines. Until recently, these substitutions were carried out through standard oligonucleotide synthesis, rather than during the SELEX procedure. Only in the past year have T7 polymerase variants become available that are capable of using 2'-OMe-substituted purines directly<sup>15</sup>.

**Applications and advantages of aptamers.** Aptamers are currently being developed as agents for a wide array of applications, including diagnostics, therapeutics, biosensors and tools for probing fundamental cellular processes (for reviews, see REFS 9, 16, 17). The broad utility of aptamers derives both from their versatility, with high selectivity and sensitivity, and their ease of screening and production. Commercial synthesis of aptamers by large-scale manufacturing is fairly uncomplicated and cost-effective; by contrast, antibodies require complex manufacturing processes using cell-based (eukaryotic or prokaryotic) expression systems. Aptamers can be thought of as 'chemical antibodies' in that they offer the advantages of antibodies — high specificity and affinity — in a relatively small, chemically synthesized molecule free from cell-culture-derived contaminants.

Functional groups such as fluorescence dyes or chemically reactive groups can be readily attached to the nucleotides during aptamer synthesis. These functional groups permit aptamers to serve as conjugates of enzymes, beads or radiotracers in diagnostic assays<sup>9,18,19</sup>. Another innovation in aptamer technology has been their use as intracellular inhibitors (so-called intramers) in which intracellular expression of aptamers can be used to probe cellular processes through binding to specific functional groups of proteins<sup>20,21</sup>.

Aptamers show high specificity, and can easily distinguish between closely related proteins<sup>22</sup> or between peptide enantiomers<sup>23</sup>. They also bind with high affinity, displaying dissociation constants in the picomolar to low-nanomolar range<sup>24</sup>. The determinants of these properties are twofold. First, aptamers are selected solely on the basis of their binding affinity, and they therefore have the potential for superior binding capabilities when compared with naturally occurring RNA or DNA molecules. Second, high-resolution, three-dimensional structural analyses have revealed that adaptive recognition (involving conformational alteration of either the protein or the aptamer) can occur, creating an even more exquisite fit between the aptamer and protein, which further increases binding affinity<sup>24</sup>.

Aptamers constitute one of four classes of oligonucleotide reagents, the others being antisense oligonucleotides, ribozymes and small interfering RNAs (siRNAs) (for a review, see REF. 25). siRNAs, discovered less than a decade ago, have generated tremendous interest as potential therapeutic agents, and several compounds have already entered clinical trials. Ribozymes and antisense oligonucleotides have been under study for more than two decades, but to date only one commercially available therapeutic agent, fomivirsen (Vitravene;

Novartis), an antisense oligonucleotide used to treat cytomegalovirus retinitis, has resulted from these approaches (for reviews, see REFS 26,27,28). Difficulties with the development of these agents include instability in biological media<sup>26</sup> and, more importantly, a requirement to cross cellular membranes in order to exert their therapeutic actions<sup>26</sup>. Like other oligonucleotide agents, aptamers are not bioavailable when administered orally and do not readily cross cell membranes. However, in contrast to these other entities, aptamers can act on extracellular targets (as do peptides and monoclonal antibodies, reagents that also have high specificity and high affinity for proteins) and are therefore suitable for use against secreted targets.

An important feature of aptamers is that they are essentially non-immunogenic even when administered in excess of therapeutic doses<sup>29,30</sup>. By contrast, peptides and monoclonal antibodies, which have therapeutic functions similar to aptamers, are often highly immunogenic. Even those monoclonal antibodies that have been largely 'humanized' by replacing much of their nonhuman component with human sequence can elicit immune responses<sup>31,32</sup>.

For these reasons, enthusiasm for developing aptamers as therapeutic agents remains strong, with many potential applications currently in the pipeline or in early developmental stages. Of these, pegaptanib is, to date, the only aptamer that has achieved FDA regulatory approval for use in a human disease. The remainder of this review will describe the events leading to that approval, including early development of an anti-VEGF aptamer with characteristics suitable for *in vivo* use, early clinical studies validating the use of pegaptanib as an anti-angiogenic agent and, ultimately, successful completion of clinical trials in patients with AMD and early clinical trials in diabetic macular oedema.

### Development of pegaptanib

**An anti-angiogenic approach targeting VEGF.** Selection of VEGF-A (also simply referred to as VEGF) as a target for aptamer development was based on its well-established role as an essential regulator in pathological angiogenesis<sup>33</sup> as occurs during tumorigenesis, ocular neovascular diseases and inflammatory conditions such as **rheumatoid arthritis**<sup>34</sup>. VEGF exerts a variety of pro-angiogenic effects through binding to two receptor tyrosine kinases, **VEGFR1** (FLT1) and **VEGFR2** (KDR)<sup>34</sup>.

VEGF was isolated independently by two laboratories, in the first instance as a powerful mediator of vascular permeability<sup>35</sup> and in the second as a potent endothelial cell mitogen and angiogenic factor<sup>36,37</sup>. The gene encoding VEGF-A contains eight exons separated by seven introns. Molecular cloning has revealed that alternative splicing of corresponding VEGF messenger RNA (mRNA) transcripts leads to the production of at least four principal isoforms containing 121, 165, 189 and 206 amino acids, respectively. VEGF<sub>165</sub>, the predominant isoform, is a heparin-binding, homodimeric 45-kDa glycoprotein that exists both in secreted and matrix-bound forms<sup>34</sup>.

Having chosen VEGF<sub>165</sub> as the target for selection of the anti-VEGF aptamer that ultimately became pegaptanib, three separate iterations of the SELEX methodology were carried out by scientists at NeXstar Pharmaceuticals<sup>13,14,33</sup>. The earliest work, reported in 1994, isolated aptamers that blocked the actions of VEGF *in vitro*, validating the potential utility of an aptamer-based approach to anti-angiogenesis<sup>33</sup>. Subsequently, the use of NH<sub>2</sub>-substituted nucleotides to increase resistance of anti-VEGF aptamers to nuclease attack was reported in 1995<sup>13</sup>, followed by the use of F-substituted nucleotides to further improve affinity<sup>14</sup>. In 1998, three stable, high-affinity candidate anti-VEGF aptamers were characterized, with one selected for development as pegaptanib<sup>14</sup>. The following is an overview of these groundbreaking studies.

**Aptamers are able to block the action of VEGF.** Jellinek *et al.*<sup>33</sup> used oligonucleotide synthesis to generate a population of 10<sup>14</sup> RNAs randomized at 30 contiguous positions and flanked by sites carrying the sequences necessary for implementing the SELEX protocol. After SELEX, cloning and sequencing yielded 37 unique sequences that fell into six related families. Competition experiments involving representatives of all six families revealed that they all bound to a similar site on VEGF; in addition, all were displaced by heparin, suggesting that the heparin-binding site was a common target of all the aptamers<sup>33</sup>. When tested on cultured human umbilical vein endothelial cells, truncated high-affinity representatives of the six families inhibited binding of VEGF to its cellular receptors in a concentration-dependent manner. These data clearly supported the concept that aptamers selected for their capacity to bind to VEGF could inhibit the binding of VEGF to its receptors *in vitro*.

**Nuclease resistance improves biological efficacy.** As mentioned previously, incorporation of modified nucleotides into aptamers increases their resistance to nucleases. During the next phase of anti-VEGF aptamer development, Green *et al.*<sup>13</sup> used SELEX to incorporate 2'-NH<sub>2</sub>-pyrimidines into aptamers and also evaluated the effects of substituting 2'-OMe for the 2'-hydroxyl of purines on nuclease resistance. One of the best aptamers obtained following SELEX had a 24-nucleotide minimal sequence for high-affinity binding; this minimal binding element was designated NX-107. Two additional modified aptamers (NX-178 and NX-213) that had phosphorothioate-linked polydeoxythymidine caps at both termini were generated from this minimal binding element; one (NX-213) had 2'-OMe purine substitution in addition to the terminal caps (TABLE 1)<sup>13</sup>. Incorporation of both of these modifications greatly improved the aptamer half-life in urine and also improved binding affinity for VEGF. These experiments demonstrated that existing technology was capable of generating an aptamer with sufficient stability in biological fluids to be useful as a therapeutic agent.

**Diabetic macular oedema**  
Thickening of the retina occurring as a result of an abnormal accumulation of fluid within the retina; a common complication of diabetes mellitus.

Table 1 | **Modifications that affect aptamer stability and affinity for VEGF**

Aptamer	Modification	Half-life in urine (hours)	Dissociation half-life	Binding affinity for VEGF ( $K_d$ , nM)	Binding affinity for PDGF ( $K_d$ , nM)	Ratio $K_d$ PDGF/VEGF
NX-107	None (minimal ligand)	1.4	NR	NR	NR	NR
NX-178	3' and 5' caps	17	12 seconds	2.4	75	31
NX-213	3' and 5' caps + 2'-OMe purine substitution	131	8 minutes	0.14	91	650

2'-OMe, 2'-O-methyl;  $K_d$ , binding affinity; NR, not reported; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor. Data taken from REF. 13.

**A higher-affinity aptamer becomes pegaptanib.** As a strategy for generating anti-VEGF aptamers with still higher affinities, Ruckman *et al.*<sup>14</sup> used 2'-F-pyrimidine-substituted RNA libraries during the SELEX process rather than the 2'-NH<sub>2</sub>-pyrimidine-substituted libraries used previously<sup>13</sup>. Although 2'-NH<sub>2</sub>-pyrimidines have been shown to decrease the stability of RNA–RNA, RNA–DNA and DNA–DNA duplexes<sup>38</sup>, 2'-F-substituted pyrimidines promote much greater thermal stability of such duplexes<sup>39,40</sup>. On theoretical grounds, this substitution could result in aptamers with higher affinities<sup>41</sup>. As a secondary consideration, oligonucleotide synthesis with F-substituted pyrimidines is more economical because protection and deprotection steps (required for NH<sub>2</sub> groups) are not needed<sup>14</sup>. Recently, it was shown that an aptamer containing both 2'-F and 2'-OMe modifications was highly stable *in vivo*, persisting much longer in the circulation than an aptamer with only 2'-OMe modification<sup>42</sup>.

After the SELEX procedure, Ruckman *et al.*<sup>14</sup> evaluated 46 2'-F-substituted aptamers that bound to VEGF<sub>165</sub>; they fell into three families with most affinities in the 5–50 pM range, representing a substantial improvement over the previously described 2'-NH<sub>2</sub>-substituted aptamer, NX-213. One candidate aptamer was selected from each of the three families for further study. After minimal sequences necessary for high-affinity binding to VEGF were defined, 2'-OMe-substitution of purines was performed chemically to increase nuclease resistance and the aptamers were terminated by a 3'-3'-linked deoxythymidine residue<sup>14</sup>. The resulting aptamers, t22-OMe, t2-OMe and t44-OMe, were characterized with respect to their stability, specificity and affinity for VEGF<sub>165</sub>, and biological functionality (TABLE 2)<sup>14</sup>.

All three aptamers demonstrated high affinity for VEGF<sub>165</sub> and also the murine homologue VEGF<sub>164</sub>, with little or no binding to VEGF<sub>121</sub><sup>14</sup>. A comparison of binding properties (TABLE 2) shows that there was little to distinguish the three candidate aptamers on the basis of their *in vitro* characterization. However, t44-OMe was the most effective at inhibiting vascular leakage from dermal microvessels following injection of VEGF into guinea pigs (Miles assay). Interestingly, one of the aptamers, t2-OMe, had no detectable activity in this assay despite having biochemical properties seemingly similar to those of t44-OMe<sup>14</sup>.

The addition of a 5'-linked 40-kDa polyethylene glycol moiety to t44-OMe further improved inhibition to 83% (despite a fourfold reduction in binding affinity for VEGF)<sup>14</sup>. This might be due to prolonged tissue residence and plasma half-life because polyethylene glycol prolongs renal clearance<sup>42</sup>. The t44-OMe polyethylene glycol-linked aptamer, having a VEGF-binding sequence of 27 nucleotides plus an additional 3'-3'-terminal deoxythymidine, was selected for further development. After interim periods in which this aptamer was designated as NX1838<sup>43</sup> and then EYE001<sup>44</sup>, it ultimately became known as pegaptanib and will be referred to as such in the remainder of this article. See FIG. 2a for the sequence and predicted secondary structure of pegaptanib.

**Inhibition of VEGF-mediated cellular responses.** Pegaptanib was further characterized in experiments with cultured endothelial cells, which express VEGFR2 at high levels and VEGFR1 at lower levels<sup>43</sup>. Pegaptanib inhibited binding of <sup>125</sup>I-labelled VEGF to human umbilical vein endothelial cells and also to human dermal microvascular endothelial cells, with 50% inhibition at a concentration of 0.75–1.4 nM. Pegaptanib also inhibited VEGF<sub>165</sub>-mediated phosphorylation of VEGFR2 and phospholipase C $\gamma$  and inhibited VEGF<sub>165</sub>-induced calcium mobilization in human umbilical vein endothelial cells<sup>43</sup>.

Although both VEGF<sub>165</sub> and VEGF<sub>121</sub> are effective mitogens for human umbilical vein endothelial cells, pretreatment of the cells with pegaptanib inhibited proliferative responses only to VEGF<sub>165</sub><sup>43</sup>. These findings are consistent with photo-crosslinking experiments that demonstrated that binding of pegaptanib to VEGF involves close contact with cysteine-137 of VEGF<sub>165</sub><sup>14</sup>. This residue is contained within the 55-amino-acid heparin-binding domain of VEGF, which is not present in VEGF<sub>121</sub><sup>34</sup>. The structure of this domain has been solved using NMR spectroscopy<sup>45</sup>; the interaction between pegaptanib (based on a model of the secondary structure; REF. 46) and cysteine-137 of the heparin-binding domain is depicted in FIG. 2b<sup>14,45,46</sup>.

**Pharmacokinetics of pegaptanib.** In subsequent work, pegaptanib was found to be stable in human plasma at ambient temperatures for more than 18 hours, demonstrating its suitability for testing in animal models<sup>47</sup>. Therefore, the pharmacokinetics of pegaptanib were

Table 2 | Comparison of candidate 2'-fluoropyrimidine anti-VEGF aptamers<sup>14</sup>

Aptamer	Length (nucleotides)	Binding affinity for VEGF ( $K_d$ , pM)	Dissociation half-life (seconds)	T <sub>m</sub> (°C)	Binding dependent on divalent cations	VEGF <sub>165</sub> IC <sub>50</sub> for VEGFR2 (M)	Miles assay (inhibition at 0.1 μM)
t22-OMe	23	72	60	49	No	$2-3 \times 10^{-12}$	13%
t2-OMe	29	130	170	66	No	$6 \times 10^{-11}$	None
t44-OMe (pegaptanib)*	27	49	90	62	Yes	$2-3 \times 10^{-12}$	48%
Scr-t44-OMe <sup>‡</sup>	27	NR	NR	NR	NR	$5 \times 10^{-8}$	None

\*Adding a 40-kDa polyethylene glycol moiety to the 5' terminus of t44-OMe generated pegaptanib which had an affinity for VEGF of 200 pM and in the Miles assay inhibited VEGF-induced permeability by 83%. †A control version of t44-OMe in which nucleotides were 'scrambled.' IC<sub>50</sub>, inhibitory concentration; NR, not reported; T<sub>m</sub>, inflection point of thermal dissociation; VEGF, vascular endothelial growth factor; VEGFR2, soluble VEGF receptor.

evaluated following a single intravenous or subcutaneous dose in rhesus monkeys (1 mg per kg). The elimination half-lives were 9.3 hours after intravenous administration and 12 hours after subcutaneous administration<sup>47</sup>. Another study that evaluated the pharmacokinetics of pegaptanib following intravenous and intravitreal injection in monkeys determined that pegaptanib was removed from the eye through plasma clearance and that biologically active pegaptanib could be detected in the vitreous humour of the eye for at least 28 days following a single 0.5-mg intravitreal dose<sup>48</sup>. No toxicities related to pegaptanib were observed following systemic or ocular administration in monkeys<sup>49</sup>. These studies laid the foundation for the subsequent clinical trials that examined pegaptanib in patients with AMD and diabetic macular oedema, ocular diseases for which a large body of evidence supports a key pathogenic role of VEGF.

#### Anti-VEGF therapy in ocular neovascular diseases

Abnormal ocular vascularization arises following several metabolic stresses that promote the production of a range of growth factors — in particular VEGF — by the retina and the retinal pigment epithelium. VEGF promotes ocular neovascularization through a number of mechanisms, and has a number of actions: as a potent endothelial cell mitogen<sup>36</sup>; a chemotactic agent for bone marrow-derived endothelial cell precursors<sup>49,50</sup>; a survival factor for endothelial cells through inhibition of apoptosis<sup>51</sup>; a promoter of blood vessel extravasation, through both upregulation of matrix metalloproteinases and decreased release of metalloproteinase inhibitors<sup>52</sup>; and a pro-inflammatory cytokine, resulting in local upregulation of intracellular adhesion molecule-1 and the consequent adhesion of leukocytes, which then amplify the entire process through secretion of yet more VEGF<sup>53,54</sup>. In addition, VEGF is a powerful vascular permeability factor, inducing vessel leakage by several mechanisms, including formation of fenestrae and dissolution of tight junctions<sup>55,56</sup>. Emerging evidence suggests that VEGF could have neuroprotective effects as well<sup>57-59</sup>.

The importance of VEGF in ocular neovascularization has been established by more than a decade of research, beginning in the early 1990s with the identification of elevated levels in several ocular neovascular syndromes<sup>60-62</sup>. These discoveries led to preclinical studies in a variety of primate and rodent models showing that

induced elevation of VEGF levels causes neovascularization and vessel leakage<sup>63-68</sup>, whereas the complementary experiment, inhibition of endogenous VEGF action through intravitreal injection of anti-VEGF antibodies or soluble VEGF receptor constructs, prevents the induction of ocular neovascularization<sup>69-72</sup>.

From the perspective of designing anti-VEGF therapies, a finding of central importance from these studies is the specific role of the VEGF<sub>165</sub> isoform in promoting leukocyte recruitment and enhanced expression of intracellular adhesion molecules that are associated with the development of ocular neovascularization<sup>54,73</sup>. In studies with a rat model, Ishida *et al.*<sup>74</sup> found that levels of both VEGF<sub>164</sub> and VEGF<sub>120</sub> (rodent equivalents of human VEGF<sub>165</sub> and VEGF<sub>121</sub>, respectively) were elevated during physiological vascularization, with a VEGF<sub>164</sub>/VEGF<sub>120</sub> expression ratio of  $2.2 \pm 1.1$ ; in the pathological neovascularization induced by ischaemia, however, this ratio increased dramatically to  $25.3 \pm 8.7$ . Pathological neovascularization was accompanied by an increased recruitment of monocytes, whose expression of VEGF mRNA was upregulated; selective monocyte depletion inhibited the pathological neovascularization induced by ischaemia while sparing physiological neovascularization. Most importantly, injection of pegaptanib inhibited the pathological neovascularization while having no effect on the physiological form, whereas injection of a VEGFR-1/Fc fusion protein, which acts as a decoy receptor for VEGF and binds to all VEGF isoforms, repressed both physiological and pathological neovascularization<sup>74</sup>.

Taken together, these studies confirm a role for VEGF in ocular neovascular diseases and demonstrate that VEGF<sub>165</sub> is the isoform largely responsible for pathological ocular neovascularization. Hence, following nearly a decade of discovery and preclinical development work led by Nebojsa Janjic at NeXagen, Inc. (which later became NeXstar Pharmaceuticals), the high-affinity, nuclease-stable anti-VEGF<sub>165</sub> aptamer — pegaptanib — was ready for human trials in AMD. Fortunately, when NeXstar was acquired by Gilead Sciences, pegaptanib was available for outlicense to Eyetech Pharmaceuticals, Inc., which then tested pegaptanib in humans in a Phase I trial<sup>44</sup>. With its safety then established, large-scale trials were initiated in patients with AMD and diabetic macular oedema.

#### Intravitreal injection

Injection of medication, air or gas into the vitreous cavity.

#### Vitreous

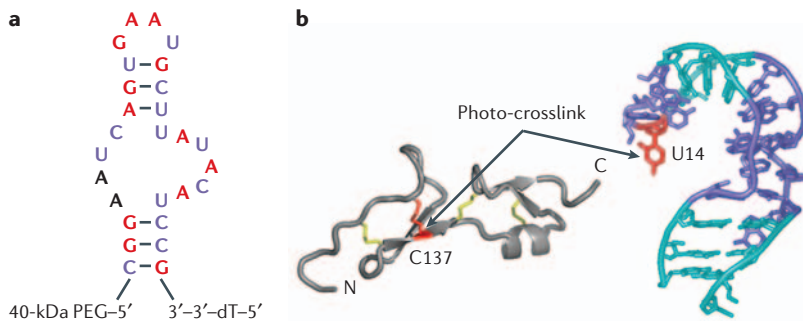
A thick, transparent, colourless, gelatinous fluid that fills the posterior segment of the eye; also known as the vitreous humour.

#### Retinal pigment epithelium

A layer of highly pigmented, phagocytic epithelial cells underlying the photoreceptors of the eye; it is a selective barrier controlling the flow of nutrients and other compounds to the retina.

#### Ocular neovascularization

A pathological condition involving the proliferation of new blood vessels in any ocular tissue.



**Figure 2 | Pegaptanib structure and target binding.** **a** | Sequence and predicted secondary structure of pegaptanib. 2'-O-methylated purines are shown in red, 2'-fluorine-modified pyrimidines are shown in blue and unmodified ribonucleotides are shown in black. The site of attachment of a 40-kDa polyethylene glycol moiety is shown. **b** | Interaction between the 55-amino-acid heparin-binding domain of vascular endothelial growth factor (VEGF)<sub>165</sub> and pegaptanib. Representation of the previously determined NMR solution structure of the free heparin-binding domain of VEGF<sub>165</sub> from Fairbrother *et al.*<sup>45</sup> is shown in grey with disulphide bonds in yellow. The aptamer (blue) is shown as a model based on the secondary structure determined by NMR<sup>46</sup>, with the helical stem regions in teal. The previously reported interaction between cysteine-137 of VEGF<sub>165</sub> (cysteine-27 of the heparin-binding domain) and uridine-14 of the aptamer<sup>14</sup> is indicated in red.

**Choroidal neovascularization**

A pathological condition involving the proliferation of new blood vessels within the choroid (the vascular layer underlying the retina).

**Macula**

The pigmented central area of the retina adjacent to the optic nerve that contains the fovea, a region of highly concentrated photoreceptor cells important for visualizing fine detail.

**Photocoagulation**

A therapy in which a light wave energy (from a laser or other light source) is used directly to coagulate (cauterize) leaky or proliferating ocular vasculature.

**Photodynamic therapy**

A therapy in which laser energy is used to activate a photosensitive compound (administered intravenously), inducing local formation of free radicals and other compounds that cause coagulation of proliferating ocular vasculature.

**Visual acuity**

A quantitative measure of optical acuity based on an assessment of one's ability to see a clearly focused image at a defined distance.

**Clinical trials of pegaptanib**

*Trials of pegaptanib in patients with neovascular AMD.*

AMD is the leading cause of blindness in people over 50 years of age<sup>75</sup>. Although there are a number of factors that contribute to the development of AMD, the principal cause of vision loss is choroidal neovascularization, a process that is particularly marked in the central region of the macula (for a review, see REF. 76). Prior to the development of pegaptanib, the only FDA-approved treatments for AMD were those that destroy abnormal ocular vessels: laser photocoagulation, which is only applicable in a small number of patients, and photodynamic therapy, a process in which an intravenously injected photosensitizing compound (see Visudyne prescribing information on the Novartis website listed in Further Information) is activated with a low-powered laser. Furthermore, the usefulness of photodynamic therapy is limited to only a subset of patients with AMD that is characterized by specific angiographic appearance.

Pegaptanib targets choroidal neovascularization, the hallmark of wet AMD, and it was therefore suggested that pegaptanib would be effective in all types of wet AMD. The safety and efficacy of pegaptanib in the treatment of choroidal neovascularization secondary to AMD were tested in two concurrent, identically designed, prospective, randomized, double-masked, multicentre, dose-ranging pivotal trials (the VEGF Inhibition Study in Ocular Neovascularization, or VISION, trials)<sup>77</sup>. In an effort to reflect the patient population usually seen by clinicians, the trial design encompassed the broadest possible inclusion criteria. Pegaptanib sodium 0.3, 1 or 3 mg by intravitreal injection or sham injection was administered every 6 weeks for 48 weeks, a total of nine treatments<sup>77</sup>. Patients receiving sham injection underwent the identical surgical preparative step as those receiving intravitreal pegaptanib and then had a needleless syringe pressed against the eye to mimic the

injection step. All injections were preceded by the subconjunctival administration of an anaesthetic and were conducted using strict ocular antisepsis. Visual assessments were made using Early Treatment of Diabetic Retinopathy Study (ETDRS) charts, originally developed for the evaluation of vision in diabetic retinopathy trials and now the accepted standard for other clinical trials in ophthalmology. At week 54, those randomized to receive pegaptanib were rerandomized (1:1) to continue pegaptanib for 48 additional weeks (eight injections) or to discontinue therapy; patients originally assigned to the sham group were rerandomized to either continue in the sham group, to discontinue sham or to receive one of the three pegaptanib doses<sup>78</sup>. The combined findings of the VISION trials presented below demonstrate that pegaptanib reduced vision loss by approximately 50% in the first year and stabilized vision in the second year<sup>77,78</sup>.

*Efficacy and safety of pegaptanib after 1 year.*

A total of 1,208 patients were randomly assigned in the two trials, and 1,186 received at least one treatment, had baseline visual acuity assessments and were included in year 1 efficacy analyses. Demographics and ocular characteristics were similar at baseline across treatment groups. In all, 7,545 intravitreal injections of pegaptanib and 2,557 sham injections were administered during year 1. Approximately 90% of the patients in each group completed the first year of the study, receiving an average of 8.5 injections out of a possible nine<sup>77</sup>.

Compared with 55% (164/296) of patients receiving sham injections, 70% (206/294) of patients receiving 0.3 mg of pegaptanib ( $p < 0.001$ ), 71% (213/300) receiving 1 mg ( $p < 0.001$ ) and 65% (193/296) receiving 3 mg ( $p = 0.03$ ) lost <15 letters of visual acuity or approximately three lines on the study eye chart between baseline and week 54 (primary efficacy endpoint). Findings with regard to other efficacy endpoints were consistent with those for the primary endpoint. Results for the 0.3 mg dose for other efficacy endpoints are presented in TABLE 3<sup>78</sup>; higher doses were not shown to provide additional clinical benefit<sup>77</sup>, and 0.3 mg is the dose that was approved by the FDA for clinical use. Mean changes in visual acuity were -8.0 letters and -15.0 letters for patients receiving 0.3 mg pegaptanib and sham, respectively ( $p < 0.0001$ ) (unpublished data, Eyetech Pharmaceuticals, Inc.). There was no evidence that sex, race, iris colour or baseline age, angiographic subtype, lesion size or visual acuity precluded a treatment benefit<sup>79</sup>.

All doses of pegaptanib were safe, and most adverse events were transient, mild to moderate in intensity and attributable to the injection procedure rather than the study drug. Across all doses, serious ocular adverse events occurred with <1% of intravitreal injections. Over the 54-week period, five cases (0.6% of the 890 patients receiving pegaptanib) each of traumatic lens injury and retinal detachment and 12 cases of endophthalmitis (1.3% of the patients injected with pegaptanib) were reported. Nine (75%) of the 12 patients with endophthalmitis remained in the trials over the 54-week period. There was no evidence of either systemic toxicity or an increased risk of potential VEGF inhibition-related adverse events<sup>77</sup>.

Table 3 | Additional year 1 efficacy endpoints in the VISION trials\*

Endpoints	Pegaptanib 0.3 mg (n = 294)	Sham (n = 296)
Maintain/gaining $\geq 0$ lines	98 (33)	67 (23)
p value versus sham	0.003	
Gaining $\geq 1$ line	64 (22)	36 (12)
p value versus sham	0.004	
Gaining $\geq 2$ lines	33 (11)	17 (6)
p value versus sham	0.02	
Gaining $\geq 3$ lines	18 (6)	6 (2)
p value versus sham	0.04	
Losing $\geq 6$ lines	28 (10)	65 (22)
p value versus sham	<0.001	
Visual acuity 20/200 or worse (legal blindness in study eye)	111 (38)	165 (56)
p value versus sham	<0.001	

\*Intention-to-treat population, n = 1,186; for missing data, the last observation carried forward method was used. Data are numbers of patients (%) unless otherwise noted; p values from the Cochran–Mantel–Haenszel test. VISION, VEGF Inhibition Study in Ocular Neovascularization. Note: this table does not present an exhaustive list of all categories, nor are all categories mutually exclusive. Adapted from REF. 78.

**Efficacy and safety of pegaptanib after 2 years.** Of patients rerandomized at week 54, 89% (941/1053) were assessed at week 102. The treatment effect of pegaptanib continued, whereas patients receiving usual care (patients who either received sham or no treatment) had the poorest visual outcomes throughout the two years. Patients rerandomized to continue 0.3 mg pegaptanib demonstrated a 45% relative benefit in mean change in vision at the end of 102 weeks compared with those receiving usual care ( $p < 0.01$ ; FIG. 3) (unpublished data, Eyetech Pharmaceuticals, Inc.)<sup>77,78</sup>. The percentage of patients gaining vision was higher among those who received 2 years of 0.3 mg pegaptanib compared with patients receiving usual care. In all, 10% of 0.3 mg pegaptanib-treated patients gained  $\geq 3$  lines of vision after 2 years compared with 6% after 1 year (unpublished data, Eyetech Pharmaceuticals, Inc.)<sup>77,78</sup>.

As in year 1, the majority of adverse events reported in the study eyes were transient, mild to moderate in intensity and attributed by investigators to the injection procedure rather than the study drug. In the 374 patients who received a second year of pegaptanib, there were four cases of retinal detachment (0.15% per injection), and no reports of endophthalmitis or traumatic cataract occurred within year 2. All doses of pegaptanib were well tolerated systemically<sup>80</sup>. There have been no signals suggestive of adverse events associated with complete VEGF blockade identified during continued monitoring of patients during the third year of the Phase III trials (unpublished data, Eyetech Pharmaceuticals, Inc.).

Although the VISION trials support the efficacy of 0.3 mg pegaptanib administered every 6 weeks, pharmacokinetic studies in humans and animals are ongoing (unpublished data, Eyetech Pharmaceuticals, Inc.) that might allow a review of dosage recommendations, with the goal being to define the lowest effective dose and the longest treatment interval.

**Year 1 visual outcomes in patients with ‘early’ lesions.** An exploratory analysis of the visual outcomes at week 54 from study patients with ‘early’ lesions was conducted. Early lesions were defined in two ways based on lesion characteristics, visual acuity and previous treatment. Results were consistent across analyses with up to 20% of patients with early lesions gaining  $\geq 3$  lines of visual acuity, and suggested that early detection and treatment with pegaptanib could result in superior vision outcomes in patients with subfoveal choroidal neovascularization secondary to AMD. This hypothesis is logical given the biology of the disease. New prospective studies are planned to assess this theory<sup>81</sup>.

**Phase II trial of pegaptanib in patients with diabetic macular oedema.** Poorly controlled diabetes can lead to ocular complications such as diabetic retinopathy and/or macular oedema (for a review see REF. 82). Diabetic macular oedema represents a leading cause of blindness in individuals between the ages of 25 and 74 in the United States (see the National Eye Institute website link in Further information). In diabetic macular oedema, vision loss occurs due to pathological processes involving micro-aneurysm formation and increased vascular permeability. Current treatment options include laser photocoagulation and vitrectomy (destruction and removal of the ocular vitreous humour)<sup>82</sup>. Although these treatments can help prevent further loss of vision, they do not improve vision and pose a risk of additional vision loss<sup>82</sup>.

Preclinical and clinical studies have confirmed that VEGF is an important regulator of diabetic retinopathy, including diabetic macular oedema and proliferative diabetic retinopathy<sup>54,60,65,83</sup>. Due to the limitations of current therapy, a clinical trial exploring the safety and efficacy of pegaptanib for diabetic macular oedema was initiated. This Phase II, randomized, double-masked, multicentre, dose-ranging, controlled, clinical trial compared sham injections or intravitreal pegaptanib (0.3, 1 or 3 mg) administered every 6 weeks for 12 weeks, with additional injections or focal photocoagulation as needed for another 18 weeks in individuals with a best-corrected visual acuity between 20/50 to 20/320 and diabetic macular oedema involving the centre of the macula<sup>84</sup>. Although the study was not powered to identify a difference in outcomes among different pegaptanib doses, pegaptanib-treated patients had improved vision, decreased oedema and an approximately 50% reduction in the need for laser therapy. The totality of the data suggested significant efficacy across a broad spectrum of patients with diabetic macular oedema.

#### Future direction with aptamers

**Improvements in ophthalmic delivery of aptamers.** Ophthalmic drugs have traditionally been administered topically, which in general provides therapeutic levels to the anterior chamber of the eye but not to the posterior segment. Therefore, topical administration of drugs has been largely infeasible for posterior segment diseases such as AMD and diabetic macular oedema. In contrast, intravitreal injection provides

#### Retinal detachment

Separation of sensory retina from the underlying retinal pigment epithelium; can result from subretinal fluid accumulation, retinal tear or retinal disease processes.

#### Endophthalmitis

Inflammation of the internal structure of the eye; in most cases the inflammation occurs as a result of infection.

#### Retinopathy

Any non-inflammatory disease of the retina; commonly describes a retinal degenerative condition resulting from impaired ocular circulation, hypoxia or systemic disease.

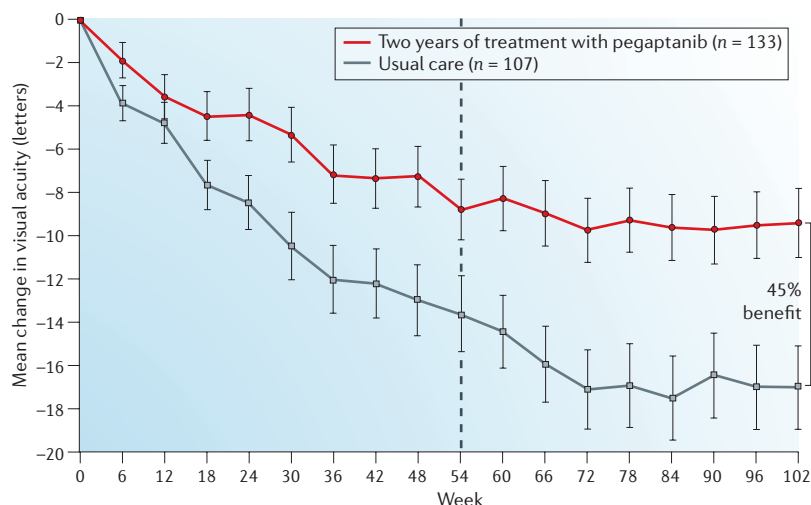


Figure 3 | Mean visual acuity throughout 2 years. Data from the VISION (VEGF Inhibition Study in Ocular Neovascularization) trials<sup>78,79</sup> (Eyetechn Pharmaceuticals, Inc., unpublished data).

direct delivery to the posterior segment and allows therapeutic levels to be attained. However, this route of administration can require repeated injections for chronic disorders and is associated with a small risk of complications<sup>85</sup>. Several alternative strategies for drug delivery have therefore been developed, such as implantable devices that deliver small, highly potent, lipophilic therapeutics intraocularly<sup>86</sup>. The small size of these implants precludes long-term (>30 days) delivery of large, water-soluble compounds, but they have been used to deliver corticosteroids<sup>86</sup>. In addition, these implantable devices generally cannot be used to deliver proteins, antibodies and other high-molecular-mass biotherapeutics<sup>87</sup>.

Aptamers, however, have been shown to be quite stable in many organic solvents and in conditions typically used in the production of polymeric formulations. Also, the presence of an amphiphilic 40-kDa polyethylene glycol moiety promotes the integration of a highly charged aptamer into the hydrophobic polymer matrix. Aptamers therefore offer unique potential for sustained delivery utilizing established polymers. Preliminary formulations consisting of pegaptanib sodium-containing poly(lactide-co-glycolide) microspheres successfully released the active drug in an *in vitro* system over a period of 24 days with near zero-order kinetics<sup>88</sup>. Recent work suggests that enhanced biodistribution can be achieved by reducing the size of the polyethylene glycol moiety from 40 kDa to 20 kDa<sup>42</sup>. This reduction in size also would allow for more efficient packaging of pegaptanib.

Administration of compounds to the eye by approaches that do not involve injection through the sclera also remain attractive alternatives to intravitreal injection. High-molecular-mass compounds such as immunoglobulins<sup>89,90</sup> and oligonucleotides as large as 24 nucleotides<sup>91</sup> have been found to be capable of diffusing through the sclera when deposited on or within the sclera. As a result, aptamers might be amenable to delivery by these alternative, less invasive methods.

#### Sclera

The tough, fibrous, white outer coat of the eye; it is continuous with the cornea and the external sheath of the optic nerve.

**Improvements in aptamer stability.** Efforts are ongoing to improve the *in vivo* stability of aptamers. One approach has been to use a T7 polymerase to generate aptamers composed entirely of 2'-OMe nucleotides, giving rise to reagents that are not only extremely nuclease resistant but also less expensive to synthesize than the 2'-OMe-containing oligonucleotides made by traditional chemical substitution<sup>15</sup>. Moreover, as 2'-OMe nucleotides are not recognized by DNA polymerases, even though they are present as natural constituents of biological systems, they have little possibility of incorporation into host DNA. This approach already has been applied to the production of a stable aptamer against VEGF<sup>15</sup>.

An elegant methodology for increasing aptamer stability has been through the use of enantiomeric (L-nucleic acid) forms known as Spiegelmers<sup>92</sup>. Because naturally occurring nucleic acids are composed of D-nucleotides, corresponding mirror-image L-oligonucleotides escape enzymatic recognition and subsequent degradation and consequently display dramatically enhanced nuclease resistance.

**New clinical indications.** Other aptamers under development, including those for cancer therapy and for anticoagulation, have entered or are about to enter clinical evaluation. AS1411 (formerly AGRO100; see the American Society of Clinical Oncology and the Antisoma websites in Further information) binds to nucleolin, a protein found within the nucleus of all cells but also uniquely expressed on the surface of tumour cells. Binding of AS1411 leads to internalization of the aptamer-nucleolin complex and a consequent antiproliferative response. Phase I trials in patients with advanced cancers have been initiated. Another agent that is in Phase I trials is ARC183, an antithrombin aptamer under development as an anticoagulant for use in coronary artery bypass grafting (see the Archemix website in Further information).

In addition to these trials with single agents, the combined administration of different aptamers is a potentially promising avenue of investigation. In preclinical studies, the combination of two small-molecule tyrosine kinase inhibitors resulted in the blockade of signalling by both VEGF and platelet-derived growth factor-B (PDGF-B), effecting a more potent inhibition of tumour growth than either agent individually<sup>93</sup>. This improved anti-angiogenic effect, leading to vessel regression, compared with inhibition of VEGF signalling alone, seems to reflect the importance of PDGF for pericyte recruitment and the provision of endothelial cell survival signals<sup>93,94</sup>. An aptamer to PDGF-B showing biological activity in preclinical studies has already been developed<sup>95-97</sup>. Combining this aptamer with pegaptanib might produce the same anti-angiogenic effect as studied above but because of their specific molecular targeting produce fewer side effects than the combination of small-molecule tyrosine kinase inhibitors, which are inherently less specific.

Finally, preclinical studies characterizing an aptamer drug-antidote pair against coagulation factor IXa have been described<sup>98</sup>. This approach, which would allow rapid reversal of anticoagulation, demonstrates a unique

advantage of aptamers over antibodies in that an antidote can be generated concurrently with the primary agent, allowing one to precisely limit its therapeutic effects.

### Conclusions

The therapeutic benefits of pegaptanib as confirmed in clinical studies in patients with neovascular ophthalmic diseases are encouraging in two respects: they provide further validation that a strategy based on understanding the underlying molecular aetiology of disease can inform the development of targeted therapies, while also providing the first example of the therapeutic application of aptamer technology. In the Phase III VISION trials, pegaptanib provided clinically meaningful and statistically significant results at all endpoints examined when compared to patients receiving usual care. Moreover, the results of the VISION trial now offer the hope of applying one unified treatment algorithm to neovascular AMD. The VISION trial data have demonstrated positive results with pegaptanib irrespective of angiographic subgroups, making the discrimination by angiography effectively irrelevant.

By showing the specific involvement of the VEGF<sub>165</sub> isoform in mediating the pathological component of ocular neovascularization, the preclinical studies of pegaptanib are likely to have broader relevance to other diseases in which aberrant production of stimulatory factors is a key component of the disease mechanism. In such cases, molecular targeting might prove to be feasible at a degree of specificity much greater than has been considered. Many proteins are produced as a variety of isoforms, and so it is likely that distinct pathological isoforms similar to that of VEGF<sub>165</sub> will be recognized in other disease contexts. Aptamers, with their exquisite specificity, are ideal agents for distinguishing these isoforms.

A theoretical advantage of this selective approach of targeting a specific pathological protein is the likelihood of a higher therapeutic index. Only longer-term studies can confirm these trends, but it seems reasonable to propose that pegaptanib, by virtue of targeting only part of the VEGF spectrum, will be less likely to cause tolerability issues than reagents that inactivate all VEGF isoforms.

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**Competing interests statement**  
The authors declare **competing financial interests**: see web version for details.

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**FURTHER INFORMATION**  
American Society of Clinical Oncology: [http://www.asco.org/ac/1,1003,12-002636-00\\_18-0026-00\\_19-001249,00.asp](http://www.asco.org/ac/1,1003,12-002636-00_18-0026-00_19-001249,00.asp)  
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