

Drug Development Series: Review

Discovery and development of anticancer aptamers

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Abstract

Aptamers, also termed as decoys or “chemical antibodies,” represent an emerging class of therapeutics. They are short DNA or RNA oligonucleotides or peptides that assume a specific and stable three-dimensional shape *in vivo*, thereby providing specific tight binding to protein targets. In some cases and as opposed to antisense oligonucleotides, effects can be mediated against extracellular targets, thereby preventing a need for intracellular transportation. The first aptamer approved for use in man is a RNA-based molecule (Macugen, pegaptanib) that is administered locally (intravitreally) to treat age-related macular degeneration by targeting vascular endothelial growth factor. The most advanced aptamer in the cancer setting is AS1411, formerly known as AGRO100, which is being administered systemically in clinical trials. AS1411 is a 26-mer unmodified guanosine-rich oligonucleotide, which induces growth inhibition *in vitro*, and has shown activity against human tumor xenografts *in vivo*. The mechanism underlying its anti-proliferative effects in cancer cells seems to involve initial binding to cell surface nucleolin and internalization, leading to an inhibition of DNA replication. In contrast to other unmodified oligonucleotides, AS1411 is relatively stable in serum-containing medium, probably as a result of the formation of dimers and a quartet structure. In a dose escalation phase I study in patients with advanced solid tumors, doses up to 10 mg/kg/d (using a four or seven continuous infusion regime) have been studied. Promising signs of activity have been reported (multiple cases of stable disease and one near complete response in a patient with renal cancer) in the absence of any significant adverse effects. Further trials are ongoing in renal and non-small cell lung cancers. In preclinical studies, additional aptamers have been described against several cancer targets, such as tenascin-C, the transcription

factor signal transducer and activator of transcription 3, and antiapoptotic and Ku proteins. [Mol Cancer Ther 2006; 5(12):2957 – 62]

Introduction

Aptamers are relatively short (12-30 base) ssDNA or RNA oligonucleotides (or peptides) that were first described from basic science studies with viruses in the 1980s (1). Derived from the Latin, “*aptus*” to fit, aptamers assume specific three-dimensional structures *in vivo*, which are able to bind with high affinity to target proteins and elicit a biological response. Although in some respects they resemble small molecules (see below), they also show highly specific binding analogous to that of an antibody to an epitope (hence their description as “chemical antibodies”; see ref. 2 for review).

Both antibodies and aptamers have been described with binding affinities in the low nanomolar to picomolar range. However, some properties of aptamers make them attractive therapeutic agents compared with antibodies. Prominent among these are their stability (they can be heated to 80°C or stored in various solvents/harsh environments and they will return to their original conformation), providing a long shelf life. Second, in studies in man, aptamers seem to lack immunogenicity (whereas antibodies are significantly immunogenic precluding repeat dosing unless they are “humanized” or produced fully human). For example, immunogenicity was found to be either absent or limited when 1,000-fold higher doses of an aptamer were given to monkeys than would be required clinically (3). In addition, interbatch variability is markedly less than with antibodies. A further advantage over antibodies is that owing to their smaller size, aptamers may penetrate tumors better and be more rapidly cleared from the blood. Chemical modifications of aptamers (as used extensively with antisense oligonucleotide-based drugs) can also be done in order, for example, to reduce their susceptibility to degradation by nucleases in serum. For example, a reduction in the nuclease degradation of aptamers has been achieved using amino or fluoro modifications at the 2' position of pyrimidines (4, 5) Finally, the biodistribution and clearance of aptamers can also be altered (e.g., tuning of plasma half-life) by the chemical addition of polyethylene glycol and other moieties (6).

A breakthrough to the more rapid discovery of aptamers that bind specifically to particular molecular targets was made using selection from libraries consisting of up to 10¹⁵ ligands by systematic evolution of ligands by exponential

Received 3/29/06; revised 9/15/06; accepted 10/13/06.

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doi:10.1158/1535-7163.MCT-06-0172

enrichment (SELEX; ref. 7). Since then, SELEX has proven to be widely applicable to generating high-affinity oligonucleotide ligands to purified biochemical targets (see ref. 8 for review) and more recently to targets residing within complex systems (such as human tumor cells; ref. 9). In contrast to antisense technologies, in which oligonucleotides target mRNA and consequently must be delivered inside the cell, aptamers can target either extracellular (thereby preventing the necessity for intracellular transportation) or intracellular proteins. Furthermore, aptamers adopt a three-dimensional structure, which may render them less susceptible to nuclease degradation (and, as with antisense oligonucleotides, may be chemically modified as described above).

Hence, aptamers possess several attractive properties compared with both antibodies and antisense oligonucleotides. Recently, the first, Macugen (pegaptanib sodium), used for the treatment of "wet" age-related macular degeneration, was approved by the Food and Drug Administration. Macugen (formerly known as NX1838) is specific for one of six isoforms of vascular endothelial growth factor, which is proposed to make it safer than providing a complete vascular endothelial growth factor blockade and is administered locally into the eye for the treatment of age-related macular degeneration. Perhaps unsurprisingly, aptamers have also been described targeting potential anticancer targets. The DNA aptamer GBI-10, which was derived using SELEX targeting a human glioblastoma cell line, bound to the extracellular tumor matrix protein tenascin-C (9). Peptide aptamers have also been described to the DNA-binding domain and the dimerization domain of the transcription factor signal transducer and activator of transcription 3 (via epidermal growth factor receptor) causing tumor cell apoptosis (10). Recently, RNA aptamers have also been described to the Ku proteins involved in DNA repair leading to a sensitization of breast cancer cells to etoposide (11) and peptide aptamers to the BCL2 antiapoptotic protein Nr-13 (12).

In the cancer arena, the most advanced aptamer and the first to enter man is AS1411, formerly developed as AGRO100 by Aptamera (Louisville, KY). AS1411 is an unmodified guanosine rich 26-mer, which anneals to form a bimolecular structure and whose anticancer properties were discovered via serendipity rather than following selection using SELEX. The remainder of this review will focus on the discovery and development of AS1411, summarize the growing body of preclinical data that supports the clinical development of AS1411, and early observations from an ongoing phase I trial.

AS1411 Mechanism of Action

As mentioned above, AS1411 was not generated using the SELEX approach as used for other oligonucleotide-based aptamers. Instead, it was developed for clinical trial based on early observations that guanosine-rich oligonucleotides (GRO) possessed antiproliferative pro-

perties against cancer cells, *in vitro*, but by a nonantisense mechanism (13). Therefore, in parallel to drug development activities (e.g., manufacture, regulatory toxicology, etc.), there has been an extensive effort to determine the primary target and underlying mechanism of action for AS1411.

Early studies revealed that the antiproliferative activity of certain GROs (such as the 29-mer GRO29A; 5'-TTTGG-TGGTGGTGGTTGTGGTGGTGGTGG-3' with a 3' amino-alkyl modification) was associated with their ability to form stable G-quartet-containing structures (13). Second, it was shown that GROs containing G-quartets bound to a specific cellular protein and, moreover, that their biological activity correlated with binding to this protein. The binding protein was detectable in both nuclear and cytoplasmic extracts and notably among proteins derived from the plasma membrane of cells. Evidence was presented suggesting that the GRO-binding protein is nucleolin (13).

Further studies showed that the primary effect of GROs on cancer cell lines is a gradual accumulation of cells in the S phase of the cell cycle, a process that occurs over several cell cycles (14). This was related to an arrest of DNA replication, possibly via modulation of helicase activity, at time points where RNA and protein synthesis was unaffected. By contrast, cells exposed to a 15-mer control GRO, which does not contain G-quartets, behaved in the same way as unexposed cells. Furthermore, a cell line derived from normal foreskin fibroblasts showed no major perturbations in the cell cycle following exposure to GRO29A. In cancer cell lines (e.g., DU145 prostate cancer), there was an evidence of GRO-induced apoptosis 72 h after exposure. In addition, in cancer cells (such as MDA-MB-231 breast cancer) at 5 days after exposure, some cells had morphology characterized by greatly enlarged nuclei and cytoplasm.

Additional studies building on those reported above for GRO29A showed that some analogues, including an unmodified DNA phosphodiester oligonucleotide (GRO29A-OH), were remarkably stable in serum-containing medium and thus resistant to nuclease degradation (15). This serum stability, observed over a 5-day period, was only seen for G-quartet-forming oligonucleotides and therefore overcomes one of the major limitations of phosphodiester-based oligonucleotides *in vivo*. Further studies confirmed that G-quartet formation is essential for biological activity of GROs (16). AS1411 is GRO29A-OH with the 3 × 5' thymidines removed.

As mentioned above, the putative initial mechanism for the antiproliferative effect of GROs, such as AS1411, is as an aptamer to cell surface nucleolin. Recent data have shed a little more light on this in revealing that AS1411 forms an intracellular complex with nuclear factor- κ B (NF- κ B) essential modulator and nucleolin and thereby inhibits activation of NF- κ B (17).

Human nucleolin is an ~76-kDa (707-amino acid) protein (although it migrates anomalously at 105 kDa by SDS-PAGE because of its charged NH₂ terminus). It is composed of an acidic histone-like NH₂ terminus, a central

domain containing four RNA-binding domains, a COOH terminus rich in RGG repeats, and four sites of phosphorylation. Nucleolin seems to be fundamental to the survival and proliferation of cells and has been suggested to play a multifunctional role involving many cellular processes, including transcription, packing, and transport of rRNA as well as replication and recombination of DNA (18). Although mainly characterized as a nucleolar protein, nucleolin also functions as a cell surface receptor where it is associated with the actin cytoskeleton and acts as a shuttling protein between cytoplasm and nucleus (19).

Over recent years, preclinical validation of cell surface nucleolin as an attractive anticancer target has emerged from several avenues. Foremost, it has been shown that nucleolin is the cell surface molecule recognized by, and responsible for, the internalization of a tumor-homing 34-amino acid peptide (F3), which binds selectively to tumors (but not skin, heart, or brain) in mice and interestingly also to tumor vasculature endothelial cells (20). A recent study has revealed that the mobilization of nucleolin to the surface of angiogenic tumor endothelial cells is mediated by vascular endothelial growth factor and nonmuscle myosin heavy chain 9 and that cell surface nucleolin may function in angiogenesis as an adhesion molecule to modulate cell matrix interaction and regulate migration (21). Furthermore, the expression of nucleolin at the cell surface was shown to correlate with the proliferative and metabolic activity of a breast cancer cell line (20). Thus, the pathophysiologic function of cell surface nucleolin may involve internalization of specific ligands. One of these is the heparin-binding growth factor midkine, a cytokine involved in growth regulation and differentiation (22). Midkine mRNA expression is also increased in various human carcinomas. A second appears to be lactoferrin, an iron-binding glycoprotein capable of inhibiting cancer cell proliferation; lactoferrin complexed to cell surface nucleolin was shown to be internalized into MDA-MB-231 breast cancer cells (23). Third, the antitumor (antiangiogenic) activity of a novel glycosaminoglycan, acharin sulphate related to heparin sulphate, has been attributed recently to binding to a phosphorylated form of nucleolin on the cell surface of cancer cells (24). Finally, it has been shown recently that extranuclear, but not nuclear, nucleolin undergoes complex O- and N-glycosylations involving specific threonines and asparagines, respectively (25).

A further role for nucleolin in protecting cells, especially leukemia cells, against apoptosis has been shown in a series of studies (26–28). In this context, nonnuclear nucleolin normally acts as an AU-rich element binding protein to mRNA coding for the antiapoptotic protein BCL2, thereby acting as a BCL2-stabilizing factor (27). However, in leukemia cells exposed to either paclitaxel or all-trans-retinoic acid, cytoplasmic nucleolin is phosphorylated, cleaved into fragments, and can no longer stabilize *bcl2* mRNA, thereby leading to apoptosis (27, 28). Recent data also show that cell surface nucleolin may act as a macrophage receptor for early apoptotic cells (29).

Although further work is required to fully elucidate the mechanism of action of AS1411, the existing data described above support a model whereby the GRO binds as an aptamer to cell surface nucleolin and is internalized, ultimately resulting in the inhibition of DNA replication and induction of S-phase cell cycle arrest (Fig. 1). *In vivo*, tumor apoptosis as well as possible antiangiogenic effects may contribute to antitumor activity, although to date, there is no reported evidence of AS1411 conferring an antiangiogenic effect *in vivo*.

AS1411 *In vitro* Growth Inhibition

GROs, including AS1411, have shown growth-inhibitory properties against a wide range of cancer cell lines, *in vitro*. Original studies showed that GRO29A (see above) at a concentration of 15 $\mu\text{mol/L}$ induced growth inhibition of prostate (DU145), breast (MDA-MB-231 and MCF-7), and HeLa cancer cell lines (13). In most cell lines, growth inhibition was clearly evident after 5 days of exposure. Further studies showed that a 10 $\mu\text{mol/L}$ concentration of either GRO29A or the unmodified GRO29AOH caused growth inhibition of the same panel of cell lines (15). Recently, we have done further studies with AS1411 to evaluate the biologically active concentration *in vitro*. The agent was incubated with a panel of human carcinoma cell lines, which included those derived from human prostate (DU145) and lung (A549) tumors. Cytotoxicity was determined by using the sulforhodamine B protein dye assay. The IC_{50} of AS1411, the concentration required to elicit cell death in 50% of tumor cells, was in the low micromolar range ($\sim 2 \mu\text{mol/L}$ following a 6-day exposure time; Fig. 2).

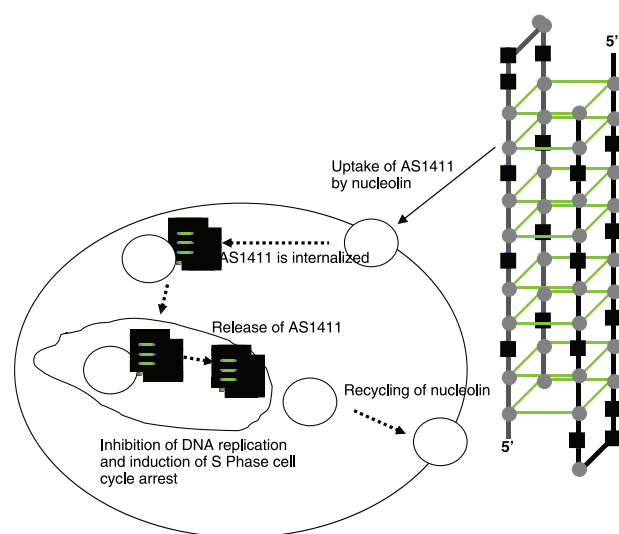


Figure 1. Structure and model for the mechanism of action of AS1411. AS1411 self-anneals into a dimer and adapts a three-dimensional structure that is conferred by eight guanosine quartets (circles). Squares, thymidine bases. AS1411 is internalized at 1 o'clock following binding to nucleolin, which is expressed on the surface of tumor cells. The mechanism of action involves inhibition of DNA replication and induction of S-phase cell cycle arrest.

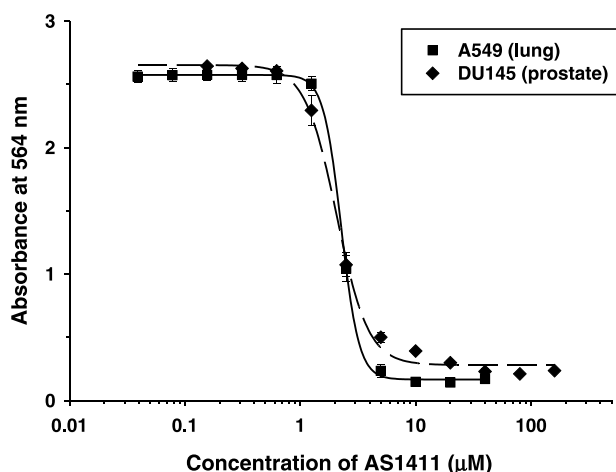


Figure 2. SRB cytotoxicity assay of AS1411 in human lung (A549) and prostate (DU145) cells. AS1411 was incubated with the cells in cell culture medium for 6 d. After 6 d, the medium was removed and replaced with 50% trichloroacetic acid. The cells were washed and incubated with sulforhodamine dye, and absorbance was measured at 564 nm. Points, mean ($n = 3$). Result is representative of three such experiments.

Of note, in these cancer cell lines, was that at least 4 days of continuous exposure of the cells to AS1411 was found to be necessary for cell death. In addition, recent studies conducted in our laboratory have shown substantial (>60%) killing of three leukemia and two of three lymphoma cell lines following a 7-day exposure to 10 $\mu\text{mol/L}$ AS1411.

AS1411 *In vivo* Biodistribution and Antitumor Activity

Biodistribution studies were done to establish an AS1411 dosing regimen that would allow plasma concentrations to be attained *in vivo* that reflect the low micromolar levels of drug required to kill cells *in vitro*. Two experimental settings were used: first, AS1411, radiolabeled with tritiated thymidine, was given to mice bearing bilateral human tumor xenografts; second, the pharmacokinetics of AS1411 was determined in rodents. Mice bearing lung and renal human tumor xenografts derived from A549 and A498 cells, respectively, were injected with a single i.v. bolus of [^3H]AS1411 (1, 10, and 25 mg/kg). Following administration of tritiated AS1411 to mice, ~63% of the radiolabel were recovered in the urine within 5 h, reflecting the initial rapid α -elimination phase. It was assumed that the agent was metabolically intact, although this remains to be experimentally confirmed using a novel reversed-phase high-pressure liquid chromatography method developed recently for the detection of AS1411 in biological fluids and tissues. Less than 1% of the injected dose was detected in the bile; therefore, it can be concluded from these data that the major route of elimination is renal. The β -phase elimination half-life of AS1411 in plasma and whole blood was 2 days and dose-related pharmacokinetics was observed. Interestingly, the tumor/blood ratios following

all three doses were 4:6 and 2:4 for the renal carcinoma and lung carcinoma xenografts, respectively (Fig. 3). These data suggest that AS1411 is accumulating in tumor tissue, although further experimental evidence is required to confirm this observation.

The pharmacokinetics of AS1411 was also investigated in rats after a single i.v. bolus, multiple injections on subsequent days, and a continuous infusion for 4 days. Maximum plasma levels of the agent were attained shortly after dosing or beginning of infusion and found to be above the limit of quantification until ~4 h after cessation of dosing. The extended half-life of this agent in plasma is in marked contrast with other unmodified phosphodiester oligonucleotides that would rapidly be degraded by serum nucleases. As described above, the stability of AS1411 in blood is probably a corollary of its ability to self-anneal and adopt a rigid confirmation conferred by eight G-quartets (16). GRO29A-OH has also been shown to be stable in serum-containing medium and cell extracts for up to 5 days and 8 h, respectively (15).

Evidence of the efficacy of AS1411 *in vivo* was ascertained from a study in nude mice bearing established s.c. human tumor xenografts derived from A549 non-small cell lung cancer cells. The agent was administered i.v. for 5 consecutive days at doses of 5, 10, and 40 mg/kg and the tumor volume was determined relative to the initial volume (Fig. 4A). Notably, administration of AS1411 resulted in a delay of tumor growth *in vivo* and a dose response can be observed, although the difference between 10 and 40 mg/kg was marginal. Antitumor effects were also observed at a dose of 10 mg/kg administered either daily for 5 consecutive days or on a Monday, Wednesday, and Friday for a 1-week regime in mice bearing A498 renal cancer, SKMES lung cancer (Fig. 4B), or MX1 breast cancer xenografts and will be reported separately. Overall, the antitumor effects of AS1411 observed to date against

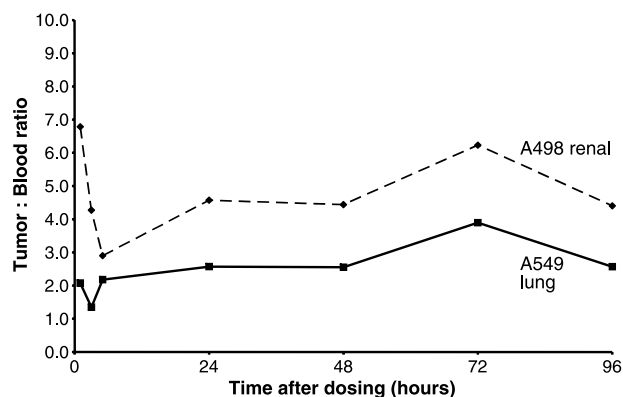


Figure 3. Tumor/blood ratios of AS1411 following administration of a single i.v. bolus (10 mg/kg) of [^3H]AS1411 to nude mice bearing A549 or A498 xenografts. Mice were subjected to terminal anesthesia and blood was removed by cardiac puncture. Tumors were excised and homogenized. The amount of radiolabeled drug in whole blood and tumor was determined by liquid scintillation counting. Points, mean ($n = 3$).

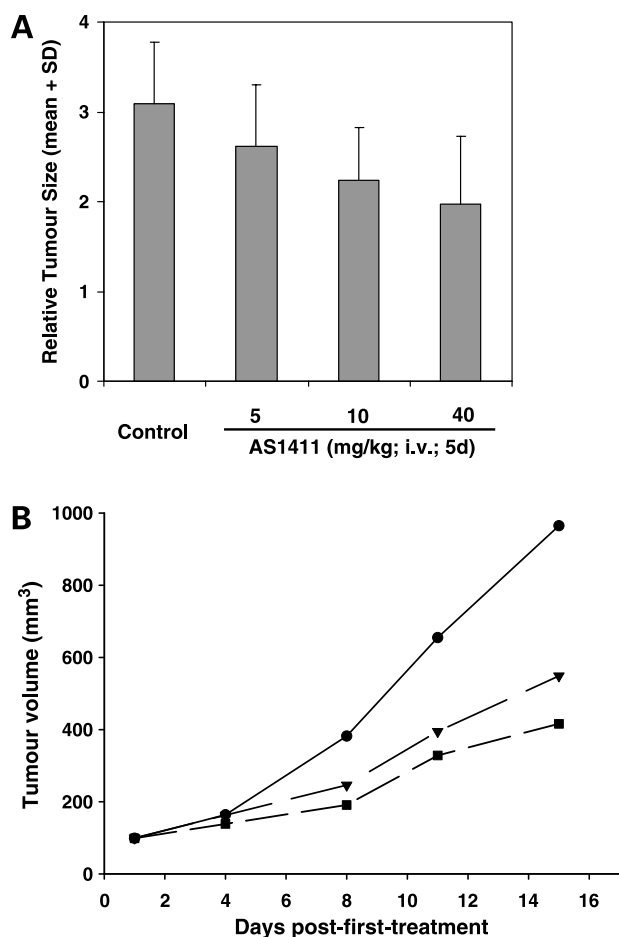


Figure 4. *In vivo* effect of AS1411 on the growth of A549 (A) and SKMES (B) human lung cancer xenografts grown s.c. in nude mice. **A**, vehicle (PBS) or AS1411 was administered daily (5, 10, and 40 mg/kg, i.v.) for 5 consecutive days. Tumor volumes were measured on day 14 and expressed as tumor volume relative to the volume on the initial day of treatment. Columns, mean ($n = 8$); bars, SD. **B**, vehicle (●), 5 mg/kg (▲), or 10 mg/kg (■) AS1411 i.p. administration on days 0, 2, and 4 from the time tumors had become established ($\sim 100 \text{ mm}^3$).

xenografts seem to result in cytostasis rather than cytotoxicity and thereby result in a delay in tumor growth.

As discussed previously, treatment of cells *in vitro* with AS1411 results in the blocking of cells in the S phase of the cell cycle. Consequently, there is a mechanistic rationale for combining the aptamer with nucleoside analogues, such as gemcitabine, which also act on cells during S-phase. Gemcitabine is the first line of treatment for pancreatic cancer and widely used in the treatment of non-small cell lung cancer. We have shown recently at least additive effects when AS1411 is added to gemcitabine in A549 cells. Furthermore, *in vivo*, the addition of AS1411 (100 mg/kg given 12 h apart on day 1, i.p.) to gemcitabine (160 mg/kg given i.p. every 3 days \times 4) to mice bearing human xenografts derived from PANC-1 pancreatic cancer cells resulted in a significant gain in antitumor activity ($P = 0.028$ on day 23 post-treatment). Further studies are

required and are ongoing to define the optimum use of AS1411 in combination with existing approved chemotherapy and will be reported separately.

AS1411 Preclinical Toxicology

The toxicology of AS1411 has been evaluated in rats (using bolus single i.v. dosing of up to 100 mg/kg) and in dogs (using a 96-hour continuous i.v. infusion regime at doses up to 10 mg/kg/d). In neither species was any significant toxicity observed, as determined by general clinical observations, clinical pathology evaluation, or gross examination of tissues at necropsy.

AS1411 Clinical Studies

Based on the original promising preclinical properties showed for GROs (e.g., growth inhibition of cancer cells *in vitro* at low micromolar concentrations) and subsequent AS1411 data (antitumor activity *in vivo* in mice bearing human tumor xenografts and a lack of significant toxicity in rats and dogs), AS1411 was selected for phase I clinical trial. Thus, AS1411 represents the first nucleic acid-based aptamer tested for the treatment of cancer in humans.

To date, results have been reported for 17 patients with advanced solid tumors (including 3 renal and 2 pancreatic) treated at the Brown Cancer Center (Louisville, KY; ref. 30). In this study, AS1411 was administered as a continuous i.v. infusion for 4 days (15 patients) or 7 days (2 patients) at doses escalated from a starting dose of 1 mg/kg/d. Patients were enrolled in cohorts of three and if no toxicity was observed up to 28 days after treatment, the dose was increased. Thus far, dose levels (mg/kg/d) of 1, 2, 4, 8, and 10 have been studied; five patients received two cycles administered over 4 days and two patients received 10 mg/kg/d administered over 7 days. No serious toxicity related to drug administration has been observed. Promising activity findings have been reported; one patient with renal cancer had a sustained partial response (at 16 months post-treatment) and there were multiple cases of stable disease at 2 months (41% of patients, including three cases sustained for 6 months or more). Full details of this phase I will be reported separately. The phase I study is continuing, focusing on patients with renal and non-small cell lung cancers. Aims are to define an optimum biological dose or dose-limiting toxicity, to gather additional human pharmacokinetic data, and to assess clinical and biological responses.

Summary

AS1411 represents the first nucleic acid-based aptamer to be tested in humans for the treatment of cancer. A phase I trial, including 17 patients, has produced promising signals in terms of both tolerability and antitumor activity. At doses of up to 10 mg/kg/d administered continuously over 7 days, AS1411 seems to be devoid of significant toxicities; these clinical data thus reflect those observed preclinically in the rat and dog. Moreover, within the context of a phase I

dose escalation trial in patients with advanced solid tumors, multiple cases of stable disease and the report of a near complete response in a patient with kidney cancer clearly warrant further clinical investigation.

There are recent reports of additional aptamers being studied in the preclinical setting as described above; it could be reasonably anticipated that further anticancer-targeting aptamers will enter clinical study over the next few years.

Acknowledgments

We thank Paula Bates, John Trent, Damian Laber, and Don Miller of the University of Louisville (Louisville, KY) for many helpful discussions and colleagues from Antisoma (London, United Kingdom), Colin Green, Hakim Djeha, Christy Ritchie, Ilijana Bajrami, David Jones, Julie Cook, Nigel Courtenay-Luck, and Gail Rowlinson-Busza for suggestions and/or technical assistance.

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