Chapter 20
Oligonucleotide Therapeutics

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20.1 Introduction

The idea of sequence-specific gene silencing by synthetic oligonucleotides targeting mRNA is at least 40 years old, but it was only in the mid-1980s when technical advances made the chemical synthesis of oligonucleotides possible that practical steps could be taken toward its implementation. The result was a deluge of experimental data in a variety of systems [1], most of which employed the phosphorothioate (PS) backbone modification, and much of which was ultimately, and unfortunately, uninterpretable.

The reason for uninterpretability is somewhat complicated. A PS oligonucleotide contains a sulfur atom that has been substituted for a nonbridging oxygen atom at each phosphorus in the oligonucleotide chain. These molecules were produced [2] because phosphodiester oligonucleotides (containing linkages identical to what is found in normal DNA) could not be used to silence gene expression either in tissue cultures or in vivo because they were very sensitive to nuclease digestion, especially to 3′-exonucleases [3] and were also rapidly cleared from the plasma through the kidneys. In contrast, phosphorothioates are degraded relatively slowly by nucleases [4] and are also cleared by the kidneys relatively slowly because of their low-affinity binding to plasma proteins (predominantly albumin) [5–7]. Further, because sulfur is immediately beneath oxygen in the periodic table, the PS linkage retains the same negative charge as the PO linkage, thus bestowing the property of extreme aqueous solubility. Importantly, the biophysical behavior of PS and PO oligonucleotides in solution are governed by their backbone charge and not by their sequence. In addition, the PS linkage retains the property of being a substrate for the RNase H, a ubiquitous, predominately nuclear enzyme that cleaves the mRNA strand of an mRNA–DNA duplex [8] and apparently functions naturally to eliminate Okazaki fragments. This ostensibly permits gene silencing to occur via

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a "pseudocatalytic mechanism" requiring only submicromolar concentrations of PS oligonucleotides for efficacy, at least when they are transfected into cells by employing carrier lipids or other vehicles. For all practical purposes, only PO and PS oligonucleotides elicit RNase H activity. (Interestingly, whether the RNAse H mechanism of gene silencing is correct or not, there is mounting evidence that it may be responsible for only a small percentage of total gene silencing. Silencing may actually occur predominately due to the activity of Ago2, the same enzyme with slicer function that cleaves the mRNA strand of an siRNA–mRNA duplex in the RISC complex) [9].

However, despite these important properties of PS oligonucleotides, which have led to their being featured in several important clinical cancer trials, the down side to this chemical modification is that PS oligonucleotides are biochemically fundamentally different from PO oligonucleotides. These differences are most significant in their ability to hybridize to a complementary mRNA strand, which is greatly diminished with respect to an isosequential PO oligonucleotide, and with respect to off-target effects, which are greatly enhanced. Diminished hybridization, reflected in a lower melting temperature (T_m) of the PS-oligo-mRNA duplex [4], directly correlates with diminished antisense activity and may be part of the reason why some clinical trials with PS oligonucleotides have not been successful, as will be discussed, although other clinically important aspects of study design and disease characteristics may also explain this observation. Fortunately, the more recent development of sugar-modified locked nucleic acids (LNAs) as antisense oligonucleotides appears to have solved the diminished hybridization problem of the PS oligonucleotide. Each LNA incorporated into a PS oligonucleotide (four are commonly employed, two at each molecular terminus) may raise the T_m of the LNA–mRNA duplex by 4–8°C [10, 11], and hence potentiate gene silencing [12–15]. Clinical trials with an anti-Bcl-2 LNA oligonucleotide in chronic lymphocytic leukemia (CLL) are currently underway, but data are not yet available.

The off-target effects of PS oligonucleotides fall into two basic categories: their ability to bind to heparin-binding proteins and their immunostimulatory properties, which can induce splenomegaly, B-lymphocyte proliferation, and cytokine production. Rodents appear to be particularly sensitive to this phosphorothioate class effect [16]. Neither category of off-target effect will be dealt with extensively in this review, but if these effects are not rigorously controlled for, particularly in experiments in immunosuppressed mice, it may be difficult, if not impossible, to differentiate sequence-specific effects from off-target effects. This has led to a great difficulty in interpreting experimental data [17], as previously noted. The problem is further compounded by the fact that the extent of immune-stimulation by PS oligonucleotides (with or without CpG motifs) in a mouse is very different from that in a human. Unfortunately, this means that preclinical animal models employing PS oligonucleotides may have had little or no predictive value for response in clinical trial patients. Nevertheless, despite these problems, some extremely interesting data have emerged from recent large, randomized, multicenter phase III trials with antisense oligonucleotides, particularly in melanoma and CLL. These trials,

in addition to a few provocative phase II–III trials in other cancers, form the basis for the majority of this review. Phase I trials and later-phase trials performed in populations other than cancer patients will not be discussed.

20.2 Clinical Trials of Oblimersen

Oblimersen (G3139 or Genasense®) is an 18-mer PS oligonucleotide that is complementary to codons 1–6 of the Bcl-2 mRNA [18] and was first synthesized approximately 15 years ago [19]. Relative to all other PS oligonucleotides of similar length studied, it is a molecule with unusual and entirely unexplained properties – its measured T_m, when in a duplex with its complementary mRNA, is substantially higher than predicted.

The molecule has been studied extensively in phase I and phase II trials. A compilation of these studies has recently been published [20]. Indeed, in the opinion of the author, sufficient data to support the antisense activity of oblimersen as the sole mechanism of action in any clinical trial is presently lacking, although the drug has demonstrated evidence of clinical activity, as described below.

Webb et al. [21] began a phase I trial with oblimersen in nine patients with lymphoma who had relapsed after at least two chemotherapy interventions and showed overexpression of Bcl-2 protein based on lymph node biopsies. Oblimersen was administered as a continuous subcutaneous infusion for 2 weeks at doses ranging from 4.6 to 73.6 mg/m^2/day. The maximum-tolerated dose (MTD) was not reached. One patient had a complete response (CR) and three patients had stable disease. Enrollment continued and a total of 21 patients were treated with oblimersen at doses up to 195.8 mg/m^2/day [22]. The MTD was 147.2 mg/m^2/day. A CR was attained in one patient, a minor response in two patients, and stable disease in nine patients. In 7 of 16 evaluable patients, Bcl-2 protein was decreased in cells from lymph nodes (two patients) and in samples of peripheral blood or bone marrow (five patients).

20.2.1 Phase III Trial of Oblimersen in Chronic Lymphocytic Leukemia

There is substantial evidence to indicate that in CLL cells, Bcl-2 silencing may lead to significant cellular apoptosis. Therefore, a randomized phase III trial of fludarabine (F) plus cyclophosphamide (C) with or without oblimersen was initiated in patients with relapsed or refractory CLL [23]. A total of 241 patients were stratified and randomized according to three criteria: responsive vs. refractory to prior fludarabine therapy, number of prior regimens (1–2 vs. ≥3), and duration of response to last therapy (>6 months vs. ≤6 months). All patients were required to have received treatment with at least one prior chemotherapy regimen that included at least two cycles of fludarabine. Using standard definitions, patients were

considered relapsed after prior treatment with fludarabine if they achieved at least a PR lasting more than 6 months; patients who did not achieve at least a PR lasting more than 6 months after their last fludarabine treatment were considered to be refractory.

In the FC group, patients received fludarabine 25 mg/m^2/day intravenously (IV) followed by cyclophosphamide 250 mg/m^2/day IV on days 1–3. In the oblimersen/FC group, patients were given oblimersen 3 mg/kg/day by continuous IV infusion on days 1–7, with FC administered at the above doses on days 5–7. Cycles were 28 days in duration, and up to six cycles were administered. The primary end point of the study was the between-treatment difference in the proportion of patients who achieved CR + nodular partial response (nPR, which is the clinical equivalent of CR; heretofore CR + nPR will be referred to as CR).

Demographic characteristics between the two groups were well balanced. The median number of prior treatment regimens in both groups was three, and previous therapy was balanced between-treatment groups [24].

Twenty patients (17%) in the oblimersen/FC group achieved a CR, as opposed to 8 (7%; $P = 0.025$) in the FC group. Moreover, these CRs were significantly more durable in the oblimersen/FC group. At 36 months of follow-up, the duration of complete remission in FC-treated patients was 22 months, whereas the median had not been reached but was estimated to exceed 36 months for patients in the oblimersen/FC group ($P = 0.031$). With 54 months of follow-up, 12/20 (60%) of oblimersen/FC patients with CR remained alive, including five who remained in complete remission [25]. Of the eight FC-treated patients with CR, only three were alive at 5 years, and all three had relapsed. Maximum benefit was seen in fludarabine-sensitive patients, e.g., those who had a PR or better for more than 6 months after prior fludarabine treatment. In this population, there was a fourfold increase in the CR rate in the oblimersen/FC group as compared to the FC control (25% vs. 6%; $P = 0.016$). With 5 years of follow-up, among all patients who achieved a response, including both complete and partial responses ($N = 103$), there was an 18-month median survival benefit for patients in the oblimersen/FC group (HR = 0.60; $P = 0.038$) [24]. In nonresponding patients, there was no difference in survival outcome between groups.

With respect to nonhematologic toxicities, nausea, pyrexia, and fatigue (primarily grade 1–2) were the most commonly occurring and affected more patients in the oblimersen/FC group than in the FC group [23]. Grade 3–4 occurrences of nausea, pyrexia, and fatigue were limited (8% vs. 2% of patients; 3% of patients in both groups; and 6% vs. 4% of patients, respectively). Importantly, in a population in which infection and immunosuppression are the most common cause of death, the incidence of grade 4 neutropenia (7% vs. 11%) was not increased with the addition of oblimersen to the FC regimen. Grade 4 thrombocytopenia was more frequent in the oblimersen/FC group, but was not associated with an increased incidence of grade 3–4 bleeding events (4% vs. 2%). In approximately 3% of patients, oblimersen administration was associated with first-cycle reactions (cytokine release with or without tumor lysis).

These data demonstrate the importance of achieving a CR for long-term survival in CLL, a point that was initially contested by the FDA. Although oblimersen has, to date, not yet been approved in this indication, the FDA is currently reconsidering that decision on the basis of the recently reported 5-year survival data.

20.2.2 Bcl-2 Silencing and Chemosensitization

While there is no doubt that oblimersen can silence Bcl-2 expression in tissue cultures, its ability to do this in vivo, and most importantly, the extent to which Bcl-2 silencing actually chemosensitizes malignant cells, as it is predicted to do, are matters of great debate. Bcl-2 is far from the only antiapoptotic protein present in the vast majority of malignant cells. Even in follicular lymphoma cells bearing the t14:18 chromosomal translocation, which produces a fused Bcl-2/immunoglobulin mRNA in about 65–70% of cases [26], it is not clear whether the Bcl-2 protein is necessary and sufficient for the maintenance of the neoplastic phenotype. In some tumors, elevated expression of Bcl-2 protein in tumor cells may merely be an epiphenomenon, despite documented clinical correlations between the expression (or "overexpression") of Bcl-2 protein and a poor prognosis in cancer patients with tumors [27–30]. Blagosklonny [31] when addressing this question, noted that Bcl-2 expression in colorectal, breast, and lung carcinomas was associated with an "increased apoptotic index, lower risk of distant metastases, and improved prognosis." Furthermore, concordant with much experimental data, cell lines may "…become resistant due to a strong selection during establishment of cells in culture, overexpression of Bcl-2 simply cannot further increase resistance and [the] effects of Bcl-2 are undetectable." Tumor cells may also become resistant to cytotoxic therapeutics by downregulating proteins in the apoptotic cascade. An example in melanoma is Apaf-1, which is downstream of Bcl-2, and in whose absence, the level of Bcl-2 protein expression would appear to be irrelevant [32].

Further complicating any potential value of Bcl-2 silencing is the role of this protein in melanoma. Its role in the pathogenesis and prognosis of clinical melanoma is controversial [33] because the protein can be found in normal melanocytes, benign nevi, and primary melanomas, in addition to melanoma metastases [34]. Interestingly, in some studies, Bcl-2 expression was decreased in melanoma cells vs. normal melanocytes [35–38]. However, this finding has not been confirmed in other studies [34, 39, 40], in which minimal differences in the expression of Bcl-2 have been observed. To add to the confusion, in advanced melanoma, about one-third of the data suggest an increase in Bcl-2 expression, while one-third suggest a decrease [33], although the function that the Bcl-2 protein actually serves, rather than just the amount of Bcl-2 protein present, would probably be more important. However, one study that was insufficiently powered has demonstrated that if lymph node deposits express Bcl-2, advanced melanoma patients have a poorer prognosis than those who do not [33]. In toto, conflicting data render the role of Bcl-2 in advanced melanoma unclear.

20.2.3 Clinical Trials in Advanced Melanoma

Dacarbazine (DTIC), the only approved chemotherapy drug for advanced melanoma, was combined with oblimersen in a phase I/II trial ($N=14$) in patients with advanced disease [41]. Oblimersen was administered via continuous IV infusion for 14 days each month. The initial dose was 0.6 mg/kg/day, increasing to a dose maximum of 6.5 mg/kg/day. Dacarbazine 200 mg/m^2 was given IV on days 5–9. Six patients also received the same total daily dose of oblimersen administered as twice-daily subcutaneous injections on days 1–7 and dacarbazine 800 mg/m^2 IV on day 5. The maximum decrease in Bcl-2 expression in patients' biopsy specimens was highly variable, and no conclusions could be drawn due to insufficient sampling. Responses included one CR, two PRs, and three minor responses, including two in patients whose disease stabilized for a period of at least 1 year.

These data led to the initiation of the largest phase III trial (GM301) in advanced melanoma to date. Between July 2000 and February 2003, 771 chemotherapy-naïve patients with advanced melanoma were randomly assigned to receive treatment with dacarbazine alone 1,000 mg/m^2/day IV for 60 min or oblimersen 7 mg/kg/day by continuous IV infusion for 5 days followed by the same dose of dacarbazine [42].

Patients were stratified according to ECOG performance status (0 vs. 1–2), presence or absence of liver metastasis, and disease site/serum LDH level. This latter category included two groups, patients with nonvisceral disease (skin, subcutaneous tissue, or lymph node disease) *and* normal LDH, and patients with visceral disease (excluding liver) *or* elevated LDH [baseline serum level more than 1.1 times the upper limit of normal (ULN)] [42]. The primary end point of the study was an intent-to-treat (ITT) comparison of overall survival between the two treatment groups. Secondary end points included progression-free survival, overall and durable response (i.e., response ≥6 months in duration), and duration of response.

The baseline characteristics of the groups were well balanced. With a minimum follow-up of 24 months, the median overall survival in the oblimersen/DTIC group was 9 months, compared with 7.8 months in the DTIC-alone group (HR = 0.87; 95% CI 0.75–1.01; $P=0.077$) [42]. Overall response rates (CRs + PRs) were 13.5% for patients treated with oblimersen/DTIC and 7.5% for patients receiving DTIC alone ($P=0.007$). Durable responses were also increased in the oblimersen/DTIC group (7.3% vs. 3.6%; $P=0.03$). Eleven patients (2.8%) in the oblimersen/DTIC group achieved a CR in comparison to three patients (0.8%) in the DTIC-alone group. Median progression-free survival was also significantly longer among patients who received oblimersen/DTIC than among those treated with DTIC (2.6 months vs. 1.6 months, HR = 0.75; $P<0.001$).

Outcome data were subsequently analyzed according to the LDH stratification category. Serum LDH has long been recognized as an important independent biomarker of poor prognosis in malignant melanoma [43] and, in the GM301 study, an interaction between treatment and baseline serum LDH was observed. Patients with LDH values ≤1.1 × ULN who received oblimersen/DTIC (approximately two-thirds [508] of the 771 subjects) were observed to have significantly better treatment

outcomes for all efficacy end points. These included overall survival (median, 11.4 months vs. 9.7 months; $P=0.02$), progression-free survival (median, 3.1 months vs. 1.6 months, $P<0.001$), overall response (17.2% vs. 9.3%; $P=0.009$), complete response (3.4% vs. 0.8%), and durable response (9.6% vs. 4.0%; $P=0.01$) [42]. On the other hand, significant differences between-treatment groups were not observed for patients with elevated baseline LDH (LDH > 1.1 × ULN). Recent data demonstrate that the extent to which pretreatment LDH level is increased, *even within the "normal" range*, is predictive of prognosis in advanced melanoma [44]. For example, a retrospective examination of data obtained from EORTC study 18951 ($N=330$) demonstrates a monotonic progression to improved prognosis in advanced melanoma patients as the value of LDH decreases, similar to what has been observed in the GM301 trial. For patients with baseline LDH ≤ 0.8 × ULN in study GM301 ($N=274$), the median survival at 24 months in the oblimersen/DTIC group vs. the DTIC group was 12.3 and 9.9 months, respectively (HR = 0.64, $P<0.001$). A confirmatory trial (AGENDA, GM307) of 300 patients, similar in design to study GM301 but with a double-blind design and limited to patients with baseline LDH ≤ 0.8 × ULN, is currently ongoing, with recruitment expected to be completed in early 2009. The results from this study should provide important prospective confirmatory data for the previously discussed observations in the GM301 trial.

But why should overall prognosis in advanced melanoma depend on pretreatment levels of serum LDH? LDH is a ubiquitous enzyme, but its expression is frequently elevated in neoplastic cells because of their shift to glycolysis secondary to relatively poor vascularization and diminished oxygen delivery. Cells dying via the process of necrosis will release LDH, but LDH is not commonly released after apoptosis. Tumor cell survival and the rate of necrosis of tumor cells may often depend on the balance between their rate of proliferation vs. the rate of vascularization of the growing tumor. Therefore, it is possible that high LDH levels in patients may reflect disease that is still growing, but is, at least in part, poorly vascularized. These types of tumors are frequently highly resistant to chemotherapy due to poor oxygen delivery, as well as possibly poor drug delivery (hence the lack of response to treatment).

Hypoxia can ultimately be an important survival factor for some tumor cells. For example, hypoxia can induce genetic instability that can select for tumor cells with increased metastatic potential [45–47] and, via c-met protooncogene activation, lead to cells that are more aggressive and invasive [48, 49]. Diminished blood flow and low pH can also compromise the functions of tumor-infiltrating immune effector cells and cytokines. Clinical studies [50] have demonstrated that the presence of hypoxic regions within tumors correlates with poor prognosis and increased metastatic risk regardless of treatment – viz., what is observed in advanced melanoma. Thus, tumor hypoxia leads to necrosis (and thus spillage of LDH) and also ultimately to more aggressive tumors and a poorer prognosis. These ideas predict that the size of the tumor is not the critical factor in either serum LDH levels or prognosis (which it was not in the GM301 trial), but rather that the balance between oxygen supply to the tumor and its intrinsic growth rate is critical.

In the GM301 trial, neutropenia and thrombocytopenia were the most significant adverse effects, but were not associated with an increase in serious infections or bleeding. In the oblimersen/DTIC group, the incidence of grade 3–4 neutropenia with infection was 4.3% for the combination vs. 2.8% for DTIC alone. Grade 1–2 bleeding events (primarily epistaxis or hematuria) were also increased in the combination-treatment group to 13.7% from 9.2% observed for the DTIC group, but more grade 3–4 bleeding events (mostly gastrointestinal) occurred with DTIC (3.1% vs. 2.2%). These rates are substantially lower than those associated with other drugs and drug regimens used for the treatment of advanced melanoma [51–53]. An increased rate of catheter-related events (venous thrombosis, infection, occlusion) was observed in the oblimersen/DTIC group (19.1% vs. 8.6%). Lower rates of adverse events resulting in treatment discontinuation or death and serious adverse events were observed in patients without elevated baseline LDH values [42].

20.2.4 Other Trials of Oblimersen

Oblimersen was added to a regimen of etoposide and carboplatin in a randomized (3:1) trial in 56 assessable patients with small-cell lung cancer [54]. In each 21-day cycle, patients in one group received oblimersen 7 mg/kg/day on days 1–8, carboplatin on day 6, and etoposide on days 6–8. Patients in the control group received the same carboplatin and etoposide regimen beginning on day 1 of each cycle. Treatment groups were balanced with respect to baseline characteristics. Response rates were nearly identical in the two treatment groups, and survival at 1 year was actually worse with oblimersen (24%, 95% CI 12–40%) than without oblimersen (47%, 95% CI = 21–73%). The incidence of grade 3–4 hematologic toxicity was also somewhat increased with the addition of oblimersen (88% vs. 60%, $P = 0.05$). The authors offer several possible explanations for the lack of improved efficacy with the oblimersen-containing regimen, one plausible explanation being that oblimersen does not adequately suppress Bcl-2 levels in patients with small-cell lung cancer, as demonstrated in the phase I study undertaken to determine the regimen for this phase II study.

In acute myelogenous leukemia (AML), Bcl-2 expression may contribute to a lower CR rate and shorter patient survival [55, 56]. In a phase I study, Marcucci et al. enrolled 29 untreated patients with AML [55]. All patients were over 60 years of age, had either intermediate or adverse cytogenetics, and initially received induction therapy with oblimersen 7 mg/kg/day by continuous IV infusion on days 1–10 + cytarabine by continuous IV infusion on days 4–10 + daunorubicin IV at one of two doses on days 4–6. CR was achieved in 14 patients (48%), and an incomplete remission was achieved in three patients (10%). Levels of normalized Bcl-2 mRNA expression in bone marrow mononuclear cells were found to be decreased from baseline ($P = 0.03$) in patients with CR, but increased from baseline ($P = 0.05$) in nonresponding patients. Expression of Bcl-2 protein in bone marrow mononuclear cells after 72 h of oblimersen demonstrated a small (about 20%), but statistically significant decrease ($P = 0.004$) in patients

with CR vs. nonresponding patients. However, given recent data that the gymnotic (i.e., naked) delivery of oligonucleotides to cells is a very slow process requiring 6 days or more to produce antisense effects, it is possible that 72 h was an insufficiently long time point for meaningful measurement of the Bcl-2 protein. All patients developed pancytopenia. Toxicities were independent of the daunorubicin dose, as well as reversible and/or "not directly attributable" to oblimersen.

A phase II trial of oblimersen + gemtuzumab ozogamicin (Mylotarg; a humanized anti-CD33 monoclonal antibody conjugated to calicheamicin) was performed in patients ≥60 years of age with AML at first relapse [57]. Oblimersen 7 mg/kg/day was administered as a continuous IV infusion on days 1–7 and 15–21, with gemtuzumab given IV on days 4 and 18. A total of 48 patients were enrolled at 18 centers, but the study was eventually terminated due to slow accrual. Based on an ITT analysis, five patients (10%) achieved a CR and seven patients (15%) achieved a CR without platelet recovery (CRp), for an overall ITT response rate of 25%. (These findings are similar to those previously reported for single-agent Mylotarg in a more favorable patient population.) [58] For the CR + CRp patients, median relapse-free survival was 3.75 months (95% CI 3.3–6.3 months), and median survival was not reached at 6 months. The probability of surviving at 6 months was 0.80, 0.86, and 0.17 for the CR, CRp, and nonresponding patients, respectively. A total of 13 patients (27%) withdrew before completing therapy, the most common reason being toxicity (6 of 13 patients). Of 16 patients who died within 30 days of last dose of study medication, five did so from treatment toxicity. Nausea was the most common nonhematologic event (79% of patients) and febrile neutropenia the most common hematologic event (50% of patients).

A Phase III trial (CALGB 10201) in which 503 untreated older patients with AML were randomized to induction treatment with cytosine arabinoside + daunorubicin followed by high-dose cytarabine consolidation therapy, with or without oblimersen 7 mg/kg/day (days 1–10 for induction, days 1–8 for consolidation) showed no differences in CR rates, overall survival, disease-free survival, or toxicity [59]. Further trials of oblimersen in AML are not planned.

Another hematologic malignancy in which oblimersen was not successful in phase III was multiple myeloma. In a phase II trial [60], 33 patients relapsing after prior chemotherapy or transplantation received oblimersen 5–7 mg/kg/day for 7 days by continuous IV infusion. On day 4, patients received dexamethasone 40 mg orally for 4 days and thalidomide 200 mg/day increasing to 400 mg/day, if tolerated, for the study duration. Responding and stable patients received maintenance dosing for up to 2 years, and the cycles were repeated every 35 days. A total of 24 of 33 patients (73% [50% historically for the combination of dexamethasone + thalidomide] [20]) had responses, including two CRs, four near CRs, 12 PRs, and six minor responses. The median duration of response was 13 months and the median overall survival was 17.4 months. A rise in polyclonal IgM (from a median of 35.5 to 94 mg/dL) was found to be predictive of response and was suggested to be due to immunostimulation by the oligonucleotide. Of seven assessable patients, three demonstrated a decrease in Bcl-2 protein in malignant cells, but there was no correlation between Bcl-2 protein levels and expression and response in this limited number of patients. The most

common grade 3 toxicities were neutropenia ($n=8$), thrombocytopenia ($n=5$), infection ($n=5$), and hypocalcemia ($n=6$). Grade 4 events were limited to neutropenia in four patients and increased serum creatinine in one patient.

The dosing scheme in the phase II trial was not pursued. Instead a randomized, multinational phase III trial of dexamethasone 40 mg/day orally for 4 days during weeks 1–3 (Cycle 1) or during week 1 (all other cycles) ± oblimersen 7 mg/kg/day by continuous intravenous infusion beginning 3 days before dexamethasone treatment in weeks 1 and 3 (Cycle 1) and in week 1 (all other cycles) was conducted in a total of 224 patients with relapsed or refractory disease [61]. The primary end point was a comparison of time to disease progression between the two groups.

At baseline, an imbalance was observed between the treatment groups in several important prognostic factors [62]. ECOG Performance Status at baseline was significantly worse in the oblimersen/dexamethasone group ($P=0.03$). In addition, more patients in the oblimersen/ dexamethasone group were categorized as having Durie–Salmon stage III, IIIa, or IIIb disease than in the dexamethasone group (70% vs. 61%, respectively). Imbalances between the two groups in baseline laboratory parameters also suggested that patients in the oblimersen/dexamethasone group were more seriously impaired than those in the dexamethasone group (ANC <1,000/mm^3: 5 and 2%, respectively; platelet count <50,000/mm^3: 7 and 3%, respectively; creatinine >2.0 mg/dL: 5 and 0%, respectively; and lactate dehydrogenase >ULN: 27 and 14%, respectively).

There was no statistically significant difference between the groups in time to tumor progression [62]. The oblimersen/dexamethasone regimen was generally well tolerated, with fatigue, fever, and nausea as the most commonly observed adverse events. Failure to show an advantage over standard treatment (dexamethasone) may be attributable to significant differences between-treatment groups at baseline that favored the dexamethasone group and/or the fact that many patients in this heavily pretreated population were refractory to dexamethasone.

20.3 OGX-011

This oligonucleotide is targeted to the mRNA of clusterin, an antiapoptotic protein that apparently promotes chemo- and radioresistance through inhibition of the function of the pro-apoptotic bax protein [63]. The compound has a phosphorothioate backbone and is further modified by the presence of 2′-methoxyethyl (MOE) substituents on the four 3′ and 5′ terminal ribose sugar moieties. The MOE modification appears to dramatically increase the tissue half-life of this oligomer, in part, by increasing its stability vs. nucleases. There is also some evidence that MOE "gap-mers" may have fewer off-target effects, in addition to diminished immunostimulatory properties. In a phase I study in combination with docetaxel [63], serum clusterin levels in the 640 mg dosing group declined approximately 35% after Cycle 1. However, declines in clusterin expression in peripheral blood mononuclear cells

could not be assessed because of the wide variability in pretreatment expression. This trial was followed by a randomized phase II trial of OGX-11 plus docetaxel vs. docetaxel plus prednisone in chemotherapy-naive patients with metastatic hormone-refractory prostate cancer [64]. Eighty-two patients at 12 centers were randomized to each arm. The docetaxel dose was 75 mg/m^2, and the OGX-11 dose was 640 mg. In the initial 56 patients, the toxicity due to OGX-011 included grade 1–2 fever and rigors in 37 and 67% of the patients, respectively. Based on a recent press report by Oncogenex, the median survival for patients in the OGX-011 arm was 27.5 months, but only 16.9 months in the control arm. This is certainly an encouraging signal with respect to proceeding to a large, phase III randomized trial in this indication.

20.4 AP 12009

AP 12009 is an antisense PS oligonucleotide targeted to the TGF-β2 mRNA. The justification for targeting TGF-β2 as an important anticancer target has been previously made by Hau et al. [65]. In brief, TGF-β2 is widely overexpressed in human tumors and is negatively correlated with prognosis. The protein blocks the proliferation and cytotoxic activity of T- and NK cells and is a potent immunosuppressant, while at the same time acting, in gliomas, as a growth and angiogenic factor. However, similar to the oblimersen story, it is unclear to what extent the in vivo mechanism of action of AP 12009 is related to these observations.

In early phase I/II trials, the drug was delivered by convection-enhanced delivery directly into the tumors of patients with grade 3 (anaplastic astrocytoma) or grade 4 (glioblastoma multiforme) disease via an implanted catheter either for four or seven days continuously. In another trial, multiple cycles of drug were administered. Twenty-four patients were enrolled, receiving a total of 48 cycles. Many of the patients had been pretreated with temozolamide. Seven showed stable disease after 28 days. One patient had a CR after one cycle of AP 12009 without further therapy; a second patient (who received a total of 12 cycles) also had a CR and was still in remission after 4.5 years.

No treatment-related deaths, grade 4 events, or catheter-related infections were observed. Two adverse events were grade 3, and the MTD was not reached after more than a 100-fold dose escalation. Plasma levels of AP 12009 after intracerebral infusion were below the limit of detection, and no laboratory abnormalities were observed. One serious event (brain edema) was considered possibly drug related. All told, the drug appeared to be extremely well tolerated [66]. A phase IIb international, open-label trial in 134 patients with high-grade (3 or 4) glioma was designed to compare (1:1:1) low (10 μM) and high (80 μM) doses of AP 12009 vs. standard chemotherapy (temozolamide or procarbazine + lomustine + vincristine) [67]. The test drug was administered weekly via convection-enhanced delivery for 6 months. Six serious adverse events possibly related to the study drug, and 37 procedure-related serious adverse events (92% grade 1 or 2) were reported. “Several long-term tumor responses were observed by local MRI reading;” response rates by central reading have not yet been presented, to our knowledge.

20.5 Affinitak

This molecule is a 20-mer PS oligonucleotide targeted to the 3′ untranslated region of the PKC-α mRNA, whose translation product was believed to be a very important signal transduction protein. The compound was evaluated in several phase I and phase II trials [68–72]. A total of 55 patients with non-small-cell lung cancer received 80 mg/m^2 cisplatin and either gemcitibine 1,000 or 1,250 mg/m^2 + Affinitak 2 mg/kg/day for 14 days via continuous IV infusion, repeated every 3 weeks. Sixteen of 48 (33%) evaluable patients achieved a response (1 CR, 15 PRs). The median overall duration of response was 7 months (95% CI 4.2–7.8 months), and the median duration of stable disease was 4 months (95% CI 3–5.5 months). Based on these data, a large, multicenter, randomized phase III trial in non-small-cell lung cancer was performed. The details of this trial have apparently not been published, but it is understood that Affinitak did not add anything to the gemcitibine + cisplatin combination, and it is no longer being clinically pursued.

20.6 Conclusions

The results of phase III studies of oblimersen in melanoma, CLL, and multiple myeloma suggest that oblimersen may not be as active in patients who have advanced disease and have received multiple prior chemotherapy regimens. Despite the evidence of clinical benefit at this point, our understanding of the mechanism of action of oblimersen, to date the only clinically active anticancer antisense oligonucleotide, is far from complete. While this is of little consequence to the advanced cancer patient, it is far from an optimal situation for those who view antisense as a platform technology. Does this mean that oblimersen is a one-off, a clinical oddity not to be repeated? Will increasing the T_m of the oligonucleotide–mRNA duplex by inclusion of LNA lead to improved clinical efficacy? Will recent advances in our understanding of the uptake of oligonucleotides by cancer cells suggest improved dosing schedules? Are siRNAs too “clean” to be active anticancer agents, and how can they be distributed efficiently to targeted cells? There are a large number of questions that need to be answered, but we believe that additional significant clinical advances can only be achieved rationally by a more complete understanding of the fundamental properties of these highly pleiotropic, biologically active compounds.

20.7 RNAi and siRNAs

The field of oligonucleotide-based therapy experienced a revival with the discovery of RNA interference (RNAi) in 1998 [73]. RNAi is a conserved endogenous mechanism, which is triggered by double-stranded (ds) RNAs leading to target-specific

inhibition of gene expression by promoting mRNA degradation or translational repression. There are two RNAi pathways that are guided either by small-interfering RNAs (siRNAs), which are perfectly complementary to the mRNA or by microRNAs (miRNAs), which bind imperfectly to their target mRNA [74]. A breakthrough in the field of siRNA therapeutic agents was achieved by Elbashir et al. [75], who demonstrated that synthetic, exogenously applied dsRNAs of 21 nucleotides in length can induce silencing in mammalian cells. In addition to the siRNA design of 21-mer duplex with 3′-overhangs at both sides, Dicer-substrate formats such as 27-mers or short hairpin (sh) RNAs have been developed that elicit a more potent gene-silencing effect at lower concentrations as compared to conventional 21-mer siRNAs [76–78].

It is remarkable how quickly after its discovery RNAi has been established as the method of choice for targeted inhibition of gene expression in mammalian systems. Because RNAi uses a natural pathway for gene silencing, it generally results in a greater potency of knockdown than antisense oligonucleotides or ribozymes. Preclinical results have confirmed the effectiveness of RNAi and have generated serious optimism about the potential for siRNA drugs. As with the other oligonucleotide-based approaches, the applications of siRNAs as a therapeutic agent face most of the above mentioned challenges. Some of these challenges, however, have already been addressed in the course of antisense oligonucleotide and ribozyme development.

Many of the standard stabilizing oligonucleotide modifications that have been already explored for antisense strategies were employed in siRNA designs. SiRNA properties can be beneficially improved by the introduction of certain chemical modifications at distinct positions in the sequence, including thermal stability of the duplex, resistance against degradation, specificity for the target mRNA, reduction of off-target effects, biodistribution, and cellular uptake [79]. In a systematic study, Jackson and coworkers reported that many individual nucleotides in the antisense strand may be modified with 2′-O-Me groups without loss of the silencing potential. A similar study has been performed with 2′-fluoro (2′-F) and 2′-O-MOE [80]. An additional advantage of using 2′-O-Me nucleotides is a reduction in off-target effects [81], as well as avoidance of the interferon responses [82]. The strategic placement of these modifications is crucial. Modifications at the 5′-end of the guide strand can inhibit the silencing effect [83], while modifications at the 5′-end of the passenger strand can improve stability as well as guide strand selection and targeting specificity [84, 85]. Incorporation of 3′-S-phophorothiolate [86], boranophosphates [87], 4′-thioriboses [88], and LNAs [89, 90], were also reported to enhance target-binding affinity and increase silencing potency.

Preclinical studies have demonstrated the safe use and the potential for therapeutic benefit of RNAi-mediated gene silencing [91, 92]. SiRNAs are in early-stage clinical trials for the treatment of viral infections, cancer, and ocular diseases. Phase I studies are planned for numerous other diseases, including neurodegenerative diseases, asthma/allergies, and inflammatory diseases [93]. The most advanced-stage testing for a siRNA-based drug is for the treatment of viral infection and was developed by Alnylam Pharmaceuticals (Cambridge, MA, USA). The siRNA

ALN-RSV01 was designed against the respiratory syncytial virus (RSV), which causes severe respiratory illness, primarily in infants [94]. The unmodified siRNAs, administered by inhalation, showed significant viral reduction in experimentally infected adult volunteers compared to the placebo group in a phase II GEMINI study and is now being tested in patients with naturally acquired RSV infection. Other examples of antiviral applications have been proposed for severe acute respiratory syndrome (SARS) [95], herpes simplex virus 2 [96], and HIV-1 [97, 98]. Serious concerns about the rapid development of drug-resistant HIV variants make the use of multiple-drug combinations inevitable. Recently, a pilot study of safety and feasibility of stem cell therapy for lymphoma patients with AIDS was initiated using a lentivirus vector encoding three anti-HIV RNAs [99]. The combinatorial approach involves a shRNA targeting tat/rev, an RNA TAR decoy, and an anti-chemokine receptor 5 (CCR5) ribozyme.

The lead product of Intradigm (Palo Alto, CA, USA) targets angiogenesis (http://www.intradigm.com) by an RNAi nanoplex particle ICS-283 comprised of a nanoparticle and two siRNAs, one against vascular endothelial growth factor (VEGF) and the other against the VEGF's main receptor (VEGFR2). The product is in preclinical development for a variety of cancer indications, and the company expects to initiate clinical evaluation in 2009. Two ongoing clinical trials also aim at angiogenesis in age-related macular deficiency (AMD). Bevasiranib (previously known as Cand5) was developed against VEGF and AGN 211745 (previously known as Sirna-027) against its receptor (VEGFR1). Early clinical studies showed that the therapeutic reagents were well tolerated and could prevent neovascularization in the eye after intravitreal injection. AGN 211745 is being investigated in a phase II study in combination with ranibizumab, and patients are currently being enrolled in a phase III study to evaluate the safety and effectiveness of bevasiranib. Controversially, a report was recently published suggesting that the suppression of neovascularization in two animal models is a generic property of siRNAs through TLR3 activation, independent of the sequence [100]. This example clearly demonstrates that preclinical studies need to be carefully conducted to prove safety and a specific siRNA-mediated silencing effect. Encouraged by earlier achievements of oligonucleotide-based therapeutics, some RNAi strategies may have been rushed into clinical trials. It is crucial to understand the basic mechanism of RNAi and its diverse related effectors to avoid toxic side effects and to develop rationally designed biopharmaceuticals.

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