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Correlation of Toxicity and Pharmacokinetic Properties of a Phosphorothioate Oligonucleotide Designed to Inhibit ICAM-1*

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ABSTRACT

ISIS 2302 is a phosphorothioate oligodeoxynucleotide with a sequence complementary to the mRNA of human intercellular adhesion molecule 1 (ICAM-1). Hybridization of ISIS 2302 to the mRNA inhibits expression of the ICAM-1 protein in response to inflammatory stimuli. A murine active antisense oligonucleotide, ISIS 3082, has been used for *in vivo* pharmacology studies and has anti-inflammatory activity in models of organ transplant rejection, ulcerative colitis, and collagen-induced arthritis at doses ranging from 0.03 to 5 mg/kg. The safety assessment for ISIS 2302 includes general toxicity studies up to 6 mo in duration in mice and monkeys, genetic toxicity studies, and reproductive/fertility studies. ISIS 3082 was examined in parallel with ISIS 2302 in mouse toxicity and reproductive studies. The toxicities observed following systemic administration of ISIS 2302 and ISIS 3082 were similar and consistent with those observed for other compounds in this chemical class and, therefore, are independent of the suppression of ICAM-1 expression. Toxicokinetic evaluation demonstrated that toxicities occurred in organs containing the highest concentrations of ISIS 2302. Evidence of immune stimulation, including dose-dependent splenomegaly, lymphoid hyperplasia, and multiorgan mixed mononuclear cell infiltrates, was the most common finding in rodent studies. Monkeys were much less sensitive than mice to immune stimulation. Kidney contained the highest concentrations of ISIS 2302. Morphologic changes observed in kidney included atrophic and regenerative changes in proximal tubular epithelium; however, there was no evidence of functional abnormalities. Additional histologic changes noted in proximal tubular epithelium included basophilic granules, which were reflective of oligonucleotide distribution and uptake in these cells. Liver also contained high concentrations of oligonucleotide, which were associated with Kupffer cell hypertrophy in mice. Changes in serum transaminases, cholesterol, and triglycerides were reflective of hepatic alterations. In monkeys, high concentrations of oligonucleotide caused a transient increase in clotting times and activation of the alternative complement pathway. All toxicities associated with ISIS 2302 were reversible and occurred at doses well above those required for pharmacologic activity or currently used in clinical trials. In addition, there has been no evidence of genetic toxicity associated with ISIS 2302, and no changes in reproductive performance, fertility, or fetal development have been noted in animals treated with ISIS 2302 or ISIS 3082.

Keywords. Toxicity; pharmacokinetics; antisense; oligonucleotide; phosphorothioate; intercellular adhesion molecule 1 (ICAM-1)

INTRODUCTION

ISIS 2302 is a representative of a novel class of potential therapeutics, the phosphorothioate oligodeoxynucleotides, which are designed to hybridize to specific mRNAs and thereby inhibit the expression of specific proteins involved in a disease process (5, 13). The nucleotide sequence of ISIS 2302 is complementary to human intercellular adhesion molecule 1 (ICAM-1) mRNA and is being tested in treatment of several inflammatory diseases, including Crohn's disease, ulcerative colitis, renal transplant rejection, arthritis, and psoriasis. Hybridization of ISIS 2302 to the target sequences results in degradation of mRNA, thereby preventing expression of ICAM-1 protein (11). ISIS 2302 has a potent and sequence-dependent capability for inhibiting ICAM-1 protein expression in human cell culture models (4). An oligonucleotide targeted to murine ICAM-1 (ISIS 3082) has exhibited pharmacologic activity in mouse models of inflammatory disease in dose ranges of 0.03–5 mg/kg (7, 27, 31–33). Inhibition of the expression of ICAM-1 during inflammatory reactions can help prevent extravasation of neutrophils from circulation into tissue and also inhibit T-cell activation (3, 6). Therefore, an antisense inhibitor of this specific adhesion molecule expression may be useful in the treatment of inflammatory disease. Preliminary evidence of safety and efficacy of ISIS 2302 in patients with Crohn's disease has been previously reported (34).

Although the specificity of antisense activity is highly selective and dependent on nucleotide sequence, the nonhybridization-dependent behavior of these compounds is very similar for members of the therapeutic class (21, 28). The phosphorothioate oligonucleotides currently examined in clinical trials are all 20–25 nucleotides in length, approximately 7,000 molecular weight, polyanionic, and resistance to nuclease degradation. These biophysical properties have greater relevance to the biological processing of these compounds by the body than to the specific nucleotide sequence. As a result, plasma pharmacokinetics, tissue distribution, metabolism, and elimination profile are similar and predictable among oligonucleotides of different sequence and different molec-

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ular targets (15, 17). Consequently, the toxicity profiles are also very similar between different nucleotide sequences. Target organs for toxicity and expected effects are also qualitatively similar. The greatest difference observed between various sequences is the potency for immune stimulation observed in rodents (29).

METHODS FOR SAFETY ASSESSMENT OF ISIS 2302

The toxicity and pharmacokinetic properties of ISIS 2302 are discussed here as representative of the phosphorothioate oligonucleotides. Although the use of antisense oligonucleotides is a novel therapeutic approach to disease treatment, the approach to safety evaluation is typical of most pharmaceutical agents. Systemic toxicity can be evaluated by intravenous, subcutaneous, intramuscular, or intraperitoneal injection. Based on the stability and tissue half-lives of these oligonucleotides, the dosing frequency can be either daily or every other day. The highest doses studied were sufficient to produce target organ toxicity (e.g., 50–100 mg/kg in mice or 10–50 mg/kg in monkeys). Typical toxicology studies performed to evaluate the tolerability of these compounds include acute toxicity studies in monkeys and 4-wk studies with a recovery phase in a rodent species and in monkeys. In addition to the standard parameters to evaluate health status of animals in these studies (i.e., body weight, hematology, serum chemistry, organ weights, and histopathology), particular attention is paid to the potential for transient inhibition of clotting times, complement activation, and hemodynamic changes, particularly in monkeys. It is also very useful to extensively characterize the toxicokinetics of these compounds as part of these studies.

One unique aspect of the safety characterization of phosphorothioate oligodeoxynucleotides is the evaluation of potential exaggerated pharmacologic effects. Because mRNA sequences may not be conserved between species, an oligonucleotide sequence specific for a human target will often not hybridize to mRNA for a given animal model. In the case of ISIS 2302, there is only a 1-base mismatch in the target region between human and monkey ICAM-1 mRNA, and ISIS 2302 is 60% as active in cynomolgus monkey aortic endothelial cells as was demonstrated in human cell culture models. However, there is much less sequence homology between mice and humans, and thus, ISIS 2302 is not active in mice. Therefore, a specific murine ICAM-1 antisense compound, ISIS 3082, was included in the mouse toxicity studies in parallel with ISIS 2302. This inclusion allows for the characterization of hybridization-independent toxicities (i.e., ISIS 2302 in mice) and of the potential toxicities associated with prolonged inhibition of ICAM-1 (i.e., ISIS 3082 in mice).

Because of the intended therapeutic applications for ISIS 2302, this oligonucleotide has been examined in 6-mo systemic toxicity studies in mice and monkeys. Reproductive, fertility, and teratology studies have also been performed in mice and rabbits.

Based on the chemical class and molecular target for phosphorothioate oligonucleotides, genetic toxicity testing has been considered an important aspect of the safety evaluation of these compounds. ISIS 2302 has been evaluated in a full range of genotoxicity assays, including the Ames bacterial gene mutation assay, the Chinese hamster ovary cell chromosomal aberrations assay, the mouse lymphoma gene mutation assay, and the *in vivo* mouse micronucleus assay.

RESULTS

Pharmacokinetics and Tissue Distribution of ISIS 2302

A basic understanding of the pharmacokinetics, absorption, distribution, metabolism, and elimination (PK/ADME) profile is important to a full understanding of the toxicity profile for phosphorothioate oligonucleotides. Plasma and tissue concentrations are dose dependent in both rodents and monkeys. Intravenous (iv) injection of ISIS 2302 is followed by relatively rapid clearance from plasma, with a half-life of approximately 30–60 min (unpublished data). Elimination from plasma is attributed to tissue distribution and to a lesser extent metabolism in plasma (1, 2, 12, 16). Very little intact oligonucleotide is excreted in urine because of the association with plasma proteins that prevent glomerular filtration. This results in a relatively high portion of each dose being distributed to tissues. Administration of ISIS 2302 by subcutaneous (sc) injection results in a slower absorption into plasma and a lower C_{max}, suggestive of a depot effect or possibly lymphatic uptake with this route of administration (15).

ISIS 2302 is widely distributed among tissues, with kidney cortex containing the highest concentrations and liver containing the highest fraction of total dose (15). Other tissues containing measurable concentrations of oligonucleotide include spleen, lymph nodes, bone marrow, lung, intestine, heart, and skin. Because the mRNA target for antisense oligonucleotides resides in cellular cytoplasm, distribution to tissues is the desired fate of oligonucleotides. Immunohistochemical studies for related oligonucleotides have confirmed the internalization of oligonucleotides into cell (9, 10). Specific cell types observed to take up particularly high concentrations of oligonucleotides include renal proximal tubular epithelium, Kupffer cells, and tissue macrophages. Although the plasma kinetics are altered by sc injection, the pattern of tissue distribution and the extent of tissue exposure at a given dose are very similar for iv and sc administration (15).

Once in tissues, oligonucleotides are cleared quite slowly. Clearance half-lives determined for phosphorothioate oligodeoxynucleotides range from 20 to 40 hr in mice and 20 to 100 hr in monkeys (14, unpublished data). Clearance from tissues appears to be primarily mediated by exonuclease degradation. The slow rate of clearance reflects the resistance to nucleases provided by the phosphorothioate modification. Because of the slow rate of clearance, there is considerable flexibility in the design of dose regimens, and daily dosing is not required to maintain tissue exposure. The primary dose regimen examined in ISIS 2302 toxicity studies was administration every other day. Comparison of daily and every-other-day dose regimens revealed comparable tissue exposure and toxicity with the same total administered dose.

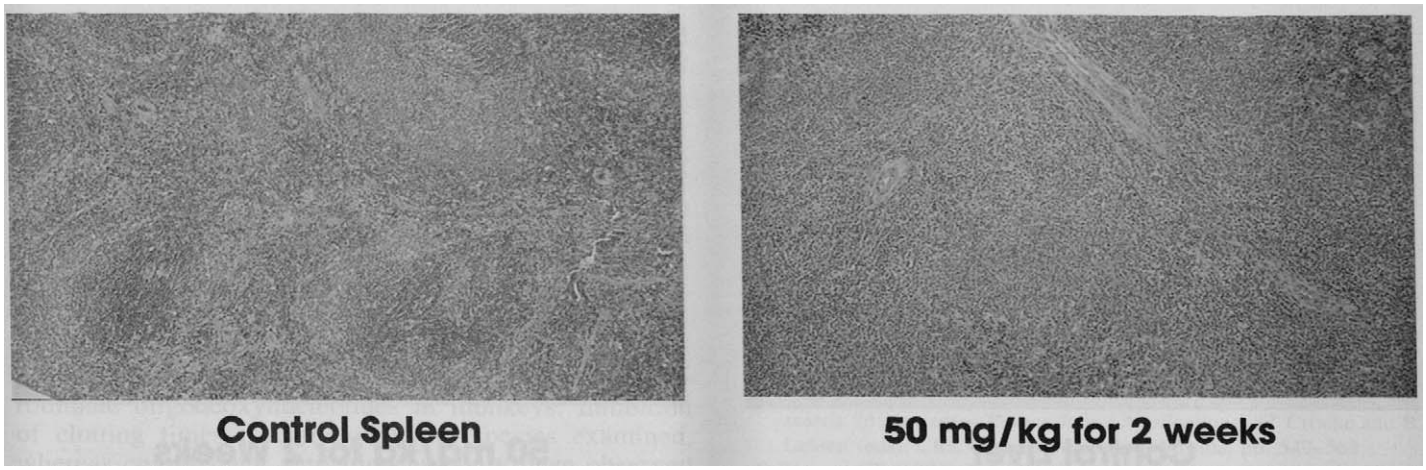

FIG. 1.—Lymphoid hyperplasia observed in mice treated with 50 mg/kg ISIS 2302 for 2 wk. Note expansion and hyperplasia of follicular and marginal zone regions of the white pulp compared to controls. H&E. ×25.

The PK/ADME properties determined in animal models, particularly for the monkey, are highly predictive of the behavior of ISIS 2302 in humans. Plasma exposure and half-life are very similar (15). Because plasma kinetics were driven by tissue distribution, it was assumed that tissue exposure would therefore also be similar. Pharmacokinetic parameters, such as clearance and volume of distribution, scale very well among the different species tested based on body weight (15). This relationship of pharmacokinetics between animals and humans is very important to the extrapolation of therapeutic and potentially toxic doses.

Toxicologic Properties of ISIS 2302

Although the pharmacokinetics and tissue distribution properties were similar across the species examined, toxicity profiles were distinct between mice and monkeys. The toxicity profile in mice was typified by evidence of dose-dependent immune stimulation, which has been observed for most phosphorothioate oligodeoxynucleotides examined (21, 28). Immune stimulation in mice treated with 20–100 mg/kg ISIS 2302 was manifested as increased spleen weight, lymphoid hyperplasia, and multiorgan mixed mononuclear cell infiltrate with fibroblast proliferation (Fig. 1) (23). Studies of related phosphorothioate oligodeoxynucleotides have revealed that these changes are also associated with increased polyclonal IgG and IgM production, B-cell hyperplasia in spleen and lymph nodes, and cytokine production (8, 26, 30). Measurement of interleukin-6, interleukin-12, and interferon-γ *in vivo* and *in vitro* is consistent with the proliferative and chemotactic alterations observed in tissue histology (24, 35). These immunostimulatory effects are reversible following cessation of treatment, and cell infiltrates are not associated with fibrosis. Although immune stimulation is considered a common property of phosphorothioate oligodeoxynucleotides, there is a spectrum of potency observed across the compounds tested. This variability appears to be due to particular sequence motifs, such as short palindromes that contain CpG dinucleotides (25, 26). The potency of ISIS 2302 for immune stimulation in mice appears to be relatively low (29). Monkeys are much less sensitive to the immunostimulatory effects of ISIS 2302 and phosphorothioate oligodeoxynucleotides in general. Repeated intravenous administration of up to 50 mg/kg for 4 wk produced no increase in spleen weight, no lymphoid hyperplasia, and no cellular infiltrates in tissues (19). Primates are not completely insensitive to immune stimulation, however; there is local erythema and edema following subcutaneous injection of these compounds, and *in vitro* exposure of human peripheral blood leukocytes to oligonucleotides does produce a mitogenic response (Isis, unpublished).

Mice also appear to be more sensitive to liver effects of ISIS 2302. In addition to the cell infiltrates, hypertrophy of Kupffer cells and dilation of sinusoids has been observed (Fig. 2). Occasional individual hepatocytes undergoing necrotic or apoptotic changes have been noted. These morphologic changes were associated with increases in aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase or decreases in cholesterol and triglycerides at 100 mg/kg in the 4-wk study (23). By comparison, the only hepatic histologic change observed in monkeys at doses ≥20 mg/kg was a slight hypertrophy of Kupffer cells, and there was no evidence of functional changes in serum chemistry parameters (19). Liver contains high concentrations of the oligonucleotide. Histochemical staining has shown that Kupffer cells in particular take up oligonucleotide, and evidence of this cell uptake can be observed on tissue sections stained with hematoxylin and eosin (H&E) as basophilic granules within the cytoplasm of Kupffer cells (9).

Morphologic changes are also observed in the kidney, which is the organ that contains the highest concentrations of oligonucleotide in mice and monkeys. The morphologic changes are generally more evident in monkeys than in mice; however, they are evident in both species at the highest doses tested. At doses of ≥10 mg/kg in monkeys, there are mild atrophic and regenerative changes in renal proximal tubular epithelial cells (19). Atrophic changes are characterized by a decrease in brush border height and apparent dilation of tubular lumen (Fig. 3).

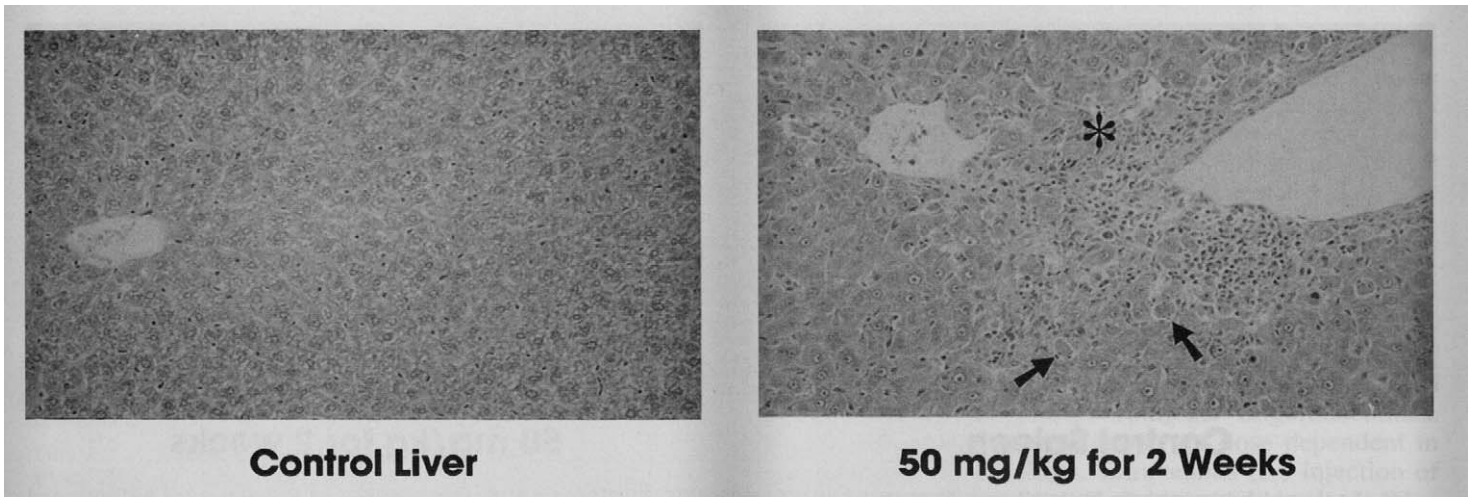

FIG. 2.—Mixed mononuclear cell infiltrate in liver of mice treated with 50 mg/kg ISIS 2302 for 2 wk. Cell infiltrates are predominantly perivascular (asterisks). Dilated sinusoids, hypertrophied Kupffer cells, and occasional degenerate hepatocytes (arrows) are also evident. H&E. ×50.

Regenerative changes are characterized by increased nucleus : cytoplasm ratio and more apparent nucleoli. Tubular resorption studies and immunohistochemical studies performed with related phosphorothioate oligodeoxynucleotides indicate that renal proximal tubular epithelial cells contain significant concentrations of oligonucleotide (9). As described for Kupffer cells, the uptake of oligonucleotide into these cells is apparent with standard H&E staining as basophilic granules within cell cytoplasm. Although morphologic changes are seen at doses as low as 10 mg/kg, there were no functional consequences of the mild atrophic or regenerative changes, even at 50 mg/kg ISIS 2302 for 4 wk (19). Work with related oligonucleotides has revealed that doses of 80 mg/kg and oligonucleotide concentrations of >3,000 μg/g are required to produce degenerative changes in renal proximal tubular epithelium that are associated with alterations in serum chemistry and urinalysis parameters (28). Atrophic and regenerative changes in kidneys of mice are less prevalent and generally are observed at doses of >50 mg/kg.

Rapid iv infusion of high doses of oligonucleotide has been associated with transient changes in clotting times and complement activation. Inhibition of clotting times is characterized by a concentration-dependent increase in APTT and to a lesser extent in prothrombin time (PT) (22). Maximal prolongation of APTT coincides with peak plasma concentration, with return to normal as oligonucleotide is cleared from plasma. Based on the rapid clearance of oligonucleotide from plasma, clotting times are generally returned to baseline within several hours of treatment. In a similar fashion, complement activation is also associated with high peak plasma concentrations of oligonucleotide (20). Activation of the complement pathway occurs through the alternative pathway, resulting in the production of biologically active complement split products, C3a and C5a. High concentrations of C5a have been associated with fluctuations in neutrophil counts and decreased blood pressure. Hemodynamic changes have been severe enough in a small fraction of monkeys to cause mortality. A threshold plasma concentration of ISIS 2302 was required for complement activation (20). Dose regimens that produce peak plasma concentrations below this threshold do not activate complement and are not

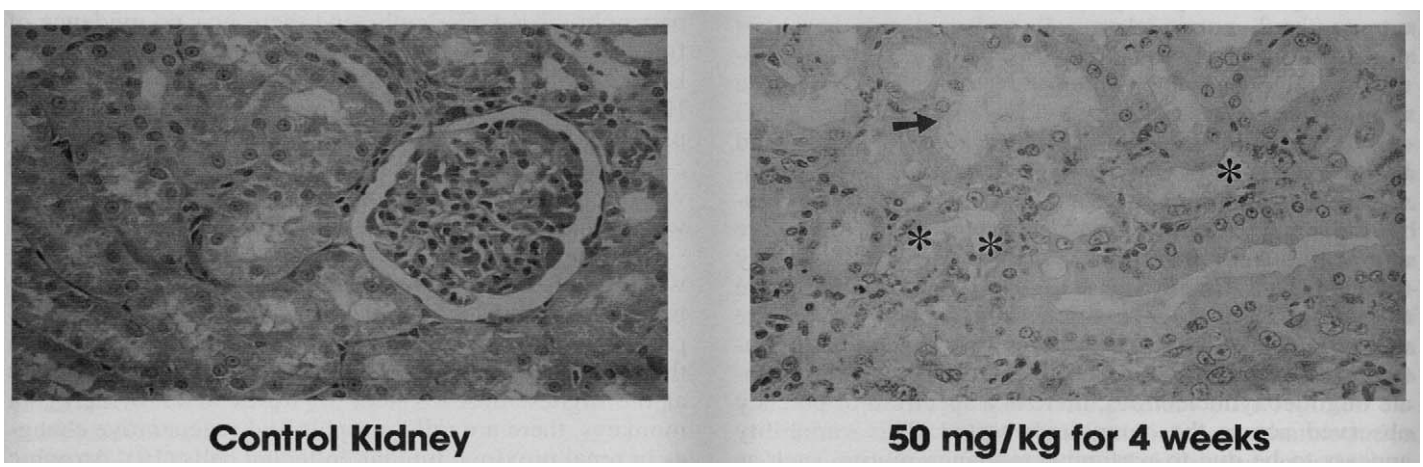

FIG. 3.—Loss of brush border within specified tubules of monkeys treated with 50 mg/kg ISIS 2302 for 4 wk (arrow). Selected proximal tubular epithelial cells also contain basophilic granular material in cytoplasm (asterisks). H&E. ×100.

associated with hemodynamic changes. The threshold plasma level for complement activation by ISIS 2302 and other phosphorothioate oligonucleotides is 40–50 μg/ml, and thus, there is a sufficient therapeutic margin for intended clinical application. As with effects on clotting times, complement activation is transient and dependent upon the continued presence of requisite drug levels in blood. Because there is no change in plasma kinetics with repeated administration of ISIS 2302, there is no accumulation or attenuation of coagulation or complement effects with repeated administration. These effects are considered class effects, and similar changes have been associated with systemic administration of other phosphorothioate oligodeoxynucleotides in monkeys. Inhibition of clotting times is common to all species examined, whereas complement activation has only been observed in monkeys thus far and has not been seen in mice/rats, guinea pigs, or dogs.

Discussion and Conclusions

The pharmacologic and toxicologic properties combine to provide an acceptable therapeutic profile for ISIS 2302. Toxicities generally occurred at doses greater than those required to produce pharmacologic effects in mouse models of inflammatory bowel disease, organ transplant rejection, and arthritis. This relationship suggests that the hybridization-dependent pharmacologic effects are of greater potency than are the hybridization-independent toxicologic effects.

The pharmacokinetic and tissues distribution properties of ISIS 2302 are also favorable for therapeutic application. Oligonucleotide was rapidly distributed from plasma to tissues, the desired site of action. Target organs for toxicity, including liver, kidney, and spleen, were also the primary sites of oligonucleotide distribution. Given the long half-lives in these tissues, considerable accumulation of oligonucleotide occurs at the dose levels required to produce morphologic changes. Toxicities associated with ISIS 2302 administration were generally fully or partially reversible following cessation of treatment.

There was no apparent difference in the toxicity profile for ISIS 2302 and ISIS 3082, suggesting no consequence of ICAM-1 inhibition in normal animals. This finding was not unexpected because constitutive expression of ICAM-1 is low, with upregulation only in response to inflammatory stimulation. The only notable differences between molecules were a decreased potency for immune stimulation and slightly greater effect on liver toxicity for ISIS 3082. These differences were unremarkable and may reflect some variability in the potency of the hybridization-independent effects of phosphorothioate oligodeoxynucleotides, particularly immunostimulation.

The safety assessment of ISIS 2302 revealed a close correlation between oligonucleotide exposure and toxicities in both plasma and tissues. Exposure to oligonucleotide was highly dose dependent. Furthermore, the pharmacokinetic parameters described in rodents and monkeys are highly consistent with the pharmacokinetic properties in humans. This consistency promotes confidence in the extrapolation of pharmacologically active and potentially toxic doses. Two-hour iv infusion of 2 mg/kg 3 times per week has been well tolerated for up to 1 mo of treatment (18). Definitive efficacy trials using this dose regimen are currently being evaluated for several inflammatory diseases. If active, this dose regimen is anticipated to have a very acceptable safety profile.

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