PHARMACOKINETICS OF AN ANTISENSE OLIGONUCLEOTIDE INJECTED INTRAVITREALY IN MONKEYS

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ABSTRACT:

The kinetics of an intravitreally administered phosphorothioate oligonucleotide, ISIS 2922, were studied in cynomolgus monkeys. Vitreal and retinal concentrations were measured after administration of 11, 57, or 115 μg/eye. ISIS 2922 concentrations in vitreous and retina were compared, after single, weekly, or biweekly doses, for potential accumulation. ISIS 2922 levels were quantified using solid-phase extraction followed by capillary gel electrophoresis. Concentrations of ISIS 2922 in the vitreous were proportional to the dose and were nearly linear with respect to the dose. The ISIS 2922 concentrations 3 days after dosing ranged from 80 nM to approximately 1.5 μM. By 14 days after intravitreal injection, the concentrations were below the limit of quantitation (<10 nM) for all dose groups. There was no accumulation in the vitreous after multiple weekly or biweekly doses. The concentrations of ISIS 2922 in the retina 2 days after a single intravitreal injection ranged from 50 nM to 1.1 μM. The uptake and disposition of ISIS 2922 in the retina appeared to have been saturated between the 57- and 115-μg doses; the average concentrations were 0.71 ± 0.24 μM (N = 4) and 0.88 ± 0.27 μM (N = 3) for the two doses, respectively. Electrophoretic profiles of extracts revealed multiple chain-shortened oligonucleotides in the vitreous and retina, suggesting extensive metabolism in both compartments. Analyses from the multiple-dose study suggested that accumulation was dependent on the total administered dose, with accumulation occurring after biweekly dosing in the 115-μg dose group and only after weekly dosing in the 57-μg dose group.

Antisense oligonucleotides are a new class of potential therapeutic agents, for treatment of human patients, that are designed to hybridize to specific mRNAs, thus inhibiting protein expression. A number of antisense oligonucleotides are currently being evaluated in multiple clinical trials as anticancer (Bayever et al., 1993), anti-inflammatory (Glover et al., 1997), and antiviral (Zhang et al., 1995) therapeutic agents. Most of the antisense compounds currently undergoing clinical trials are phosphorothioate oligodeoxynucleotides, i.e., DNA molecules in which a single nonbridging oxygen has been replaced by a sulfur (Agrawal et al., 1991). The replacement of this single nonbridging oxygen by a sulfur dramatically increases in vivo stability (Sands et al., 1994). By targeting genetic information, the antisense approach provides a level of selectivity not available with traditional therapeutic agents (Dean and McKay, 1994; Dean et al., 1994). Antisense oligonucleotides have shown specific pharmacological activity in a number of animal models (Crooke and Bennett, 1996).

ISIS 2922 is an antisense phosphorothioate oligonucleotide (21 nucleotides in length) that has shown antiviral activity against human CMV,2 by inhibition of expression of the major immediate-early gene (Azad et al., 1993). ISIS 2922 has recently undergone evaluation, in phase III clinical trials, as a treatment for CMV-induced retinitis (Hutcherson et al., 1995; Lieberman et al., 1997; Boyer et al., 1997; Palestine et al., 1994, 1995). Human CMV is a ubiquitous herpesvirus that can cause severe morbidity or death in immunocompromised patients and is often associated with gastroenteritis and sight-threatening chorioretinitis. All currently available treatments for CMV-induced retinitis target inhibition of the CMV DNA polymerase; therefore, cross-resistance is common. ISIS 2922 targets a specific mRNA and inhibits the expression of a pivotal replication protein of one of the immediate-early genes (Azad et al., 1993). The target for ISIS 2922 is a 21-nucleotide sequence in the coding region. Thus, ISIS 2922 inhibits CMV replication at a different target than do currently available treatments, and viruses resistant to those treatments do not show cross-resistance to ISIS 2922.3 Therefore, ISIS 2922, as an antisense oligonucleotide therapeutic agent, not only has a different molecular target than do currently available treatments for CMV-induced retinitis but also may be less likely to result in the rapid emergence of resistance (Azad et al., 1995), making it a useful therapeutic alternative.

We previously reported that ISIS 2922 was cleared from the vitreous, after single intravitreal injections in rabbits, with first-order kinetics (Leeds et al., 1997). Clearance from the vitreous appeared to result primarily from distribution to other ocular tissues, whereas uptake and clearance from the retina appeared to be related to metabolism and other clearance mechanisms (Leeds et al., 1997). The estimated half-lives for elimination of ISIS 2922 from the vitreous and retina of rabbits were 62 and 79 hr, respectively. Pharmacokinetic modeling predicted that weekly induction doses followed by biweekly

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2 Abbreviations used are: CMV, cytomegalovirus; RPE, retinal pigmented epithelial; SPE, solid-phase extraction.

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maintenance doses would cause some retinal accumulation with the induction (weekly) regimen but no significant accumulation with the biweekly maintenance dosing regimen. This modeling also predicted that the concentration in the retina resulting from that dosing regimen would range from 5.0 to 0.6 mM throughout the treatment period, well above the in vitro EC50 of 0.1 μM (Azad et al., 1993).

In the present study, the pharmacokinetic parameters and metabolism of ISIS 2922 were studied after intravitreal injections into cynomolgus monkeys. Monkeys were given intravitreal injections of 11, 57, or 115 μg of ISIS 2922, equivalent to an initial intravitreal concentration (based on an average vitreal volume of 1.5 ml) of 1, 5, or 10 μM, respectively. Concentrations of ISIS 2922 in the vitreous humor and retina were then determined 2, 7, and 14 days after the injection, using gel-filled capillary electrophoresis. Additional monkeys were given multiple doses of ISIS 2922 either every week or every other week, to determine the potential for accumulation using these dosing regimens, as well as to determine the potential correlation between any observed ocular toxicities and the vitreous and retinal oligonucleotide concentrations. These studies also provided an opportunity to use animal models to determine the relationship between dose and tissue concentrations, which provided a basis for dose and regimen selection for clinical trials.

Materials and Methods

Synthesis and Purification of ISIS 2922. ISIS 2922 was chemically synthesized by standard phosphoramidite reactions, using 1,2-[3H]benzodithiol-3-one during the oxidation step (Iyer et al., 1990). The compound was purified by preparative reverse-phase and anion-exchange chromatography and was then formulated in a sterile bicarbonate buffer, pH 8.7, at concentrations of 2.3, 1.15, and 0.23 mg/ml.

In Vivo Study. The monkeys in this study were obtained and cared for in accordance with all applicable federal and state guidelines. The use of monkeys in this study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. ISIS 2922 was administered by intravitreal injection (0.05 ml) to cynomolgus monkeys, to evaluate ocular pharmacokinetics. Doses of 11, 57, and 115 μg of ISIS 2922 were injected, to yield estimated vitreal concentrations of 1, 5, and 10 μM ISIS 2922, respectively. Pharmacokinetic evaluations of vitreous and retina were performed at each dose level after single doses, as well as with weekly and biweekly multiple-dose schedules. For the single-dose study, two monkeys were examined at each time point for each dose. In the multiple-dose arm of the study, one monkey (two eyes) was examined at each dose and time point. Ophthalmic examinations were performed on all available eyes on dosing and tissue-collection days. Examinations were performed with the aid of an indirect ophthalmoscope and a biomicroscope. Vitreous and retina were collected from eyes at scheduled necropsy, weighed, frozen, and submitted for bioanalytical evaluation.

Oligonucleotide Extraction. Retinal tissues were weighed, mixed with internal standard [a 27-mer (T) oligonucleotide] in proteinase K digestion buffer (2.0 mg/ml proteinase K in 20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40), and homogenized (Crooke et al., 1996). Overnight incubation at 37°C was followed by phenol/chloroform extraction. Retinal extracts as well as vitreous samples were then purified by sequential SPE (anion-exchange SPE followed by reverse-phase SPE) (Leeds et al., 1996). Samples were further desalted by membrane dialysis before analysis using gel-filled capillaries (Leeds et al., 1996). Vitreous samples were diluted with SPE buffer and extracted immediately by strong anion-exchange SPE, after addition of a known concentration of the T1/2 internal standard. The quantitation of the oligonucleotides was based on the vitreal volume or retinal weight extracted and the initial T1/2 concentration.

Capillary gel electrophoresis separations were performed using a Beckman P/ACE capillary electrophoresis instrument (model 5010) with a 27-cm column (effective length, 20 cm) containing 12% polyacrylamide, with 8.3 M urea in 100 mM Tris-borate, pH 8.5, as the running buffer. Separation was achieved at 50°C and 550 V/cm. Oligomers eluting from the column were detected by UV absorption at 260 nm. The limit of quantitation for ISIS 2922 in vitreous and retinal samples was estimated to be 10 nM.

Pharmacokinetic Analyses. Half-lives for ISIS 2922 in the vitreous and retina were estimated using logarithmic-linear regression from lines that best fit the concentration data. AUC values were estimated using the linear trapezoidal rule.

Results

In Vivo Studies. After treatment on day 1, ocular inflammation was documented in 10–15% of the eyes on day 3. Inflammation was observed only in the 11- and 57-μg dose groups. Inflammatory changes were indicative of cyclitis and anterior uveitis. In the more severe cases of cyclitis, inflammatory cells and protein were deposited in the vitreous body, making it difficult to observe the fundus with the ophthalmoscope. The inflammatory process appeared to subside somewhat by day 7. The decision was made on day 8 to institute prophylactic corticosteroid treatment, by administering triamcinolone acetonide (40 mg/ml; E.R. Squibb Co., Princeton, NJ) subconjunctivally at a dose of 7–8 mg/eye. Subconjunctival steroid administration resulted in marked reduction of active cyclitis and anterior uveitis. Corticosteroids were administered to all eyes on all remaining treatment days. Ocular inflammation was effectively controlled by steroid treatment and was seen only very sporadically after the last dose on day 29. Ophthalmic abnormalities noted in several animals given repeated intravitreal injections of ISIS 2922 included retinal cell infiltrates and perivasculitis. These changes were noted in all groups, but the incidence increased with dose. There was also an observation of retinal pigmented epitheliopathy on day 31 (2 days after the last dose) in two monkeys. One animal that had received three doses of 115 μg demonstrated RPE changes in both eyes; the other animal treated with 115 μg showed no visible lesions on day 31 or 42. RPE alterations were noted in one eye of an animal treated with 57 μg but were limited to the periphery and were milder than with the higher dose. RPE changes may be secondary to the inflammatory changes noted above.

Metabolism. After the injections, ISIS 2922 and chain-shortened metabolites were detected in the retina and vitreous of all eyes beginning at the first time point (2 days after the injection) (figs. 1 and 2). An additional, more slowly migrating peak was seen in retinal extracts (fig. 1). The pattern of metabolite appearance over time was consistent with exonuclease activity and the removal of a single terminal nucleotide, except for the more slowly migrating peak. At the earliest time point examined (2 days after administration of the dose), the predominant peak in both retinal and vitreous extracts was the parent oligonucleotide (figs. 1A and 2A). At 7 days after the injection, the full-length oligonucleotide was still the predominant peak but more chain-shortened oligonucleotides were present in the retina (fig. 1B). In the vitreous there appeared to be fewer metabolites, but the concentrations were so low that only the largest metabolites and the parent oligonucleotide were observed (fig. 2B). At 14 days after administration of the dose, the amount of the parent compound in the retina was roughly equivalent to the levels of most other metabolites observed (fig. 1C). At that time point, no measurable oligonucleotide was found in the vitreous at any dose level (fig. 2C, table 1). The electrophoretic profiles demonstrated that the percentage of total oligonucleotide detected as full-length ISIS 2922 decreased with time after injection in the retina. In retinal extracts, the chain-shortened metabolites appeared to be present in higher proportions than seen in the vitreous. However, the concentrations of ISIS 2922 and metabolites in the vitreous 1 week after intravitreal administration were quite low and smaller oligonucleotides, if present, were likely below the...
limit of detection. The presence, in both the vitreous and retinal compartments, of multiple chain-shortened oligonucleotides suggested that metabolism was active in both compartments or, alternatively, that oligonucleotides moved freely between the vitreous and the retina.

**Concentrations of ISIS 2922 and Total Oligonucleotide in the Vitreous after a Single Dose.** Concentrations of ISIS 2922 in the vitreous measured 2 days after a single dose were linearly proportional to dose (fig. 3A). The mean vitreous concentrations ranged from $0.11 \pm 0.03 \mu M (N = 4)$ at the low dose to $1.28 \pm 0.36 \mu M (N = 3)$ at the high dose (table 1). There was more variability in measured oligonucleotide levels in samples from different eyes from the same time point than would be expected based only on differences in vitreous volume or on assay variability (Leeds et al., 1997). This variability is consistent with either substantial differences in clearance rates, slight differences in dissection, or slight differences in injection techniques. Additionally, variations resulting from inflammation are not known. Samples in which the concentrations measured were $>2$ SD from the mean value determined for the other three values in the single-dose study were not included in the calculation of the mean values. Two days after the injection, when the first samples were taken, the mean concentrations of ISIS 2922 were approximately 11, 13, and 12% of the theoretical concentrations estimated from the dose amount and the average vitreous volume (1.5 ml) for the 11-, 57-, and 115-μg doses, respectively. The concentrations of ISIS 2922 decreased from the first time point onward, so that the concentrations in the vitreous samples from the low-dose group were close to the limit of detection by 7 days after the injection, precluding analysis at later time points.

**Concentrations of ISIS 2922 and Total Oligonucleotide in the Retina after a Single Dose.** Unlike in the vitreous, retinal concentrations of ISIS 2922 did not increase linearly with dose. The ISIS 2922 concentration in the retina 2 days after a single 115-μg dose was only 24% higher than that seen after the 57-μg dose. The mean ISIS 2922 concentrations were 0.12, 0.71, and 0.88 μM after doses of 11, 57, and 115 μg, respectively (table 1). The best curve fit to the data was logarithmic ($r^2 = 0.77$), rather than linear ($r^2 = 0.67$) (fig. 3B). As was seen in the vitreous, the ISIS 2922 concentrations decreased after the first time point. By the second time point of the study (7 days after the injection), the mean retinal concentrations of ISIS 2922 had decreased to 0.028, 0.158, and 0.44 μM for the 11-, 57-, and 115-μg doses, respectively. These decreases represented 4-fold decreases in concentration for the 11- and 57-μg dose groups but only a 2-fold decrease in the ISIS 2922 concentration for the high-dose group, suggesting that clearance processes were saturated at the highest dose. By the last time point (14 days after the injection), the concentrations of ISIS 2922 had decreased substantially in all dose groups (table 1).

In the retina, there was more rapid disappearance of the parent drug than of metabolites, suggesting either that the retina is more metabolically active than the vitreous or that clearance of metabolites from the retina occurs at a slower rate than does metabolism. At the first time point examined (2 days after the intravitreal injection), the percentage of full-length oligonucleotide was greater in the vitreous than in the retina at all three concentrations (table 1). Between 2 and 7 days after the intravitreal injection, the total detected oligonucleotide levels in the retina decreased for the low- and middle-dose groups, whereas the
concentration of total detected oligonucleotide increased for the high-dose group; the mean concentration of total detected oligonucleotide increased between the two time points from 1.89 to 2.20 μM. Therefore, in addition to what appears to be saturation of uptake into the retina between the middle and high doses, it appears that clearance mechanisms for oligonucleotides were saturated between the same two doses, resulting in slower clearance at the highest dose.

**Pharmacokinetic Parameters of ISIS 2922 in Vitreous and Retina.** The half-lives of ISIS 2922 in the vitreous were estimated to be 24 and 22 hr for the 57- and 115-μg doses, respectively. Because concentrations in the vitreous decreased rapidly and only two time points showed levels above the limit of detection, elimination half-lives of ISIS 2922 in the vitreous were estimated using the initial theoretical concentration after the injection, to obtain three time points on which to base the half-life estimation (fig. 4A). Data from rabbits suggest that this estimation yields values close to the actual vitreal concentrations (Leeds et al., 1997). Because ISIS 2922 concentrations in the 11-μg dose group were so close to the limit of detection at the second and third time points, a half-life was not estimated for this group. The half-lives in the retina were estimated using concentrations determined at three time points in the retina (fig. 4B). The estimated elimination half-life in the retina approximately doubled, from 45 to 78 hr, as the dose was increased from 57 to 115 μg. The estimated elimination half-life in the vitreous appeared to be independent of dose, whereas the elimination half-life in the retina appeared to increase between the 57- and 115-μg doses. This confirms the observation that clearance from the retina was saturated at the highest dose.

The AUC values were calculated using the linear trapezoidal rule (table 2). Although the number of data points available for this calculation was limited, the estimated AUC values in both the vitreous and retina increased proportionally with dose, indicating that, although peak retinal concentrations did not increase linearly with dose, total exposure did.

**Potential for Accumulation of ISIS 2922 in the Retina and Vitreous after Multiple Administrations.** To understand the intravitreal pharmacokinetics of these compounds in primates and to plan for optimal exposure in clinical trials, different dosing regimens were examined to determine their respective potentials for accumulation of oligonucleotide. Mean retinal concentrations were determined 2 days after a single dose, three doses administered every other week, or three doses administered every week followed by one dose 2 weeks later (fig. 5). At the 115-μg dose the retina accumulated ISIS 2922 even with biweekly administration, whereas at the 57-μg dose accumulation was seen only with weekly administration of the compound. These results are consistent with the longer elimination half-life at 10 μM and suggest that optimal retinal exposure can actually be attained at the middle dose (57 μg), without saturating clearance mechanisms in the retina.

**Discussion**

In this study the concentrations of ISIS 2922 and total detected oligonucleotides were determined in vitreous and retinal extracts after
intravitreal administration of three different doses, the range of which encompassed that being used in phase III clinical trials. Although phosphorothioate oligonucleotides injected intravitreally are distributed to ocular tissues other than retina (Leeds et al., 1997), only the retinal concentrations were measured because the site of pharmacological action is the retina.

The oligonucleotide parent ISIS 2922 and multiple chain-shortened metabolites were measured using gel-filled capillary electrophoresis. Metabolites formed by the partial oxidation of the thioate linkages on the backbone of the phosphorothioate oligonucleotide were not resolved by capillary electrophoresis. Mass spectroscopic studies, however, have indicated only limited oxidation of the thioate backbone of phosphorothioate oligonucleotides (Gaus et al., 1996). Therefore, analysis of the samples for the presence of chain-shortened metabolites should have provided an adequate summary of the metabolic fate of this compound.

The presence of multiple chain-shortened metabolites in both the vitreous and retina suggests that metabolism was active in both compartments. Alternatively, metabolism might have occurred in the retina alone, with diffusion of metabolites from the retina into the vitreous. This possibility cannot be eliminated. However, the existence of a slowly migrating metabolite unique to retinal extracts (fig. 1) suggests that diffusion from retina to vitreous did not occur to a significant degree. The most abundant metabolites at early time points in both the vitreous and retina were compounds one or two nucleotides smaller than the parent oligonucleotide, ISIS 2922. Because exonucleases remove single nucleotides from the 5’ or 3’ end of a nucleic acid, the sequential formation of smaller oligonucleotides indicates that exonucleolytic activity predominated over endonucleolytic activity. These results are consistent with the results from other studies, which also indicated that exonucleolytic cleavage was the primary route of metabolism of phosphorothioate oligonucleotides (Gaus et al., 1996; Crooke et al., 1996).

The concentrations found in the vitreous were linear with respect to dose 2 days after single intravitreal injections of the three concentrations. In addition to the linear relationship between dose and intravitreal concentration, the estimated elimination half-lives after the 57- and 115-µg doses were nearly equivalent and were estimated to be 24 and 22 hr, respectively. In a previous study of ISIS 2922 in New Zealand white rabbits, the elimination half-life from the vitreous was first-order, as was observed here, but it was much longer (approximately 62 hr) (Leeds et al., 1997). Although there were few time points available for the half-life estimates in this study, the half-life appears to be very different from that observed in rabbits. This difference between the elimination half-lives for rabbits and monkeys is not understood but may be related to differences in retinal vascularization. Rabbit retinal vascularization is through a single central vessel (Conrad and Robinson, 1977; Gelatt, 1991), whereas monkey eyes, similar to human eyes, are highly vascularized. Alternatively, the difference in elimination half-lives may be related to the fact that outflow from the aqueous humor via the uveoscleral pathway is greater in primates than in rabbits (Wilkie and Wyman, 1991). Smaller molecules being used to treat human CMV-induced retinitis, which are administered directly into the vitreal compartment, have also been reported to have long elimination half-lives. The elimination half-life for (S)-1-(3-hydroxy-2-phosphorylmethoxypropyl)cytosine was estimated to be 18 hr in rabbits (Dolnak et al., 1992). The elimination half-life estimates for intravitreally administered ganciclovir in humans ranged from 8.1 to 18.3 hr (Jabs et al., 1987; Henry et al., 1987; Morlet et al., 1996). ISIS 2922 appears to have a longer elimination half-life in the vitreous than do lower-molecular weight compounds currently being used to treat CMV-induced retinitis. This slow elimination of the oligonucleotide from the vitreous may afford the retinal cells, which are in constant contact with vitreal fluid, more time to take up the phosphorothioate oligonucleotide.

The concentrations in the retina at the earliest time point (2 days after the dose) were not linear with respect to dose. Rather, the retinal concentration appeared to plateau between the 57- and 115-µg doses. Therefore, a 2-fold higher dose resulted in an only 24% difference in the mean concentrations. Although it cannot be said with certainty that this time point was the time of the maximal concentration, previous experience in rabbits suggested that this time point was close to the time of the maximal concentration for the retina (Leeds et al., 1997). The estimated elimination half-life in the retina increased from 45 to 78 hr between the 57- and 115-µg doses, suggesting saturation of clearance mechanisms. In fact, the concentration of total detected oligonucleotides increased between the first and second time points in the high-dose group, confirming the apparent saturation of clearance.

**TABLE 2**

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<th>Dose</th>
<th><em>AUC in vitreous and retina after intravitreal injection of ISIS 2922</em></th>
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<td><strong>Vitreous</strong></td>
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*µM-hr = (µg/7122)/kg)-hr.
mechanisms. The estimated half-life for the 57-μg dose (45 hr) was shorter than the 79-hr half-life seen in the rabbit study for a comparable dose (Leeds et al., 1997), and this shorter half-life may be related to the vascularization or outflow differences in rabbits and monkeys mentioned above.

In an effort to understand the dynamics of oligonucleotide movement from the vitreous to the retina, AUC values were estimated for both tissues. The results suggest that exposure in the retina increases linearly with dose, even though the peak concentrations in the retina do not. This appears to be a consequence of the saturation of clearance at the highest dose. An additional consequence of the saturation of clearance is the increased potential for accumulation after multiple doses. The level of accumulation was also dose and schedule dependent. Weekly doses of 57 μg caused accumulation of ISIS 2922 in the retina, whereas biweekly doses did not. Biweekly doses of 115 μg did cause retinal accumulation of ISIS 2922.

Retinal concentrations of ISIS 2922 were high enough to maintain in vitro antiviral activity for 1 week after a single intravitreal dose of either 57 or 115 μg. If the concentrations of the N–1 metabolites (which have nearly 40% of the antiviral activity of the parent oligonucleotide) (Azad et al., 1993) were included, the level of antiviral activity 1 week after intravitreal injection would be slightly higher. ISIS 2922 metabolites smaller than N–1 have virtually no antiviral activity (Azad et al., 1993).

In summary, intravitreal administration of ISIS 2922 results in retinal concentrations sufficient to inhibit CMV replication in vitro. The retinal ISIS 2922 concentrations, because of slow metabolism and clearance, remain in the concentration range of antiviral activity for >1 week. The retinal concentrations of ISIS 2922 were nearly equivalent after intravitreal administration of either 57- or 115-μg doses. The apparent saturation of clearance mechanisms at the higher dose and the decreased potential for accumulation after multiple doses at the lower dose suggest that the lower dose of 57 μg may provide nearly equivalent therapeutic concentrations of ISIS 2922 in the retina, compared with the higher dose.

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References