Delivery of NADPH-Cytochrome P450 Reductase Antisense Oligos Using Avidin−Biotin Approach

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Although avidin-mediated intracellular delivery of oligonucleotides or proteins has been shown before, the efficacy studies are lacking. Here, we tested the effectiveness of avidin for delivery of a cytochrome P450 reductase (CPR) antisense oligo in rat liver epithelial cells. A phosphorodiamidate morpholino oligo (PMO) against CPR was biotinylated using four reagents with short, cleavable, or long linkers, followed by conjugation with avidin. The dose-inhibitory response of the unmodified PMO in the presence of a transfection reagent (Endoporter, EP) and the effectiveness of the EP-assisted and avidin-assisted delivery of biotinylated PMOs were tested by Western blot analysis. Additionally, in a preliminary study, the avidin−biotin PMO with a long linker was also tested in vivo in rats. The biotinylated oligos were at least as effective as the unmodified oligo. Whereas the avidin conjugate of biotinylated PMO with the short linker was ineffective, those with the long linkers showed significant reductions in CPR protein expression. Finally, the in vivo study showed modest, but significant, reductions in CPR activity. In conclusion, these studies show for the first time that avidin-mediated intracellular delivery of biotinylated oligos can effectively knock down target genes in vitro, depending on the length of the linker. Additionally, the avidin−biotin approach may be of potential value for in vivo gene knockdown.

NADPH-cytochrome P450 reductase (CPR) is a flavoprotein first identified in the liver by Horecker in 1950 (*1*) as a cytochrome c reductase. The key function of CPR is to donate electrons mainly to cytochrome P450 enzymes. It has been reported (*2*−*5*) that CPR can generate reactive oxygen species (ROS) by direct and indirect mechanisms. The direct mechanisms include the generation of superoxide (*3*) and redox cycling of cellular Fe^{3+} (*2, 3*) and quinones/quinoid compounds (*5*) to generate hydroxyl radicals and free semiquinone radicals, respectively. Additionally, CPR contributes to generation of superoxide and hydrogen peroxide indirectly via uncoupling mechanism of the cytochrome P450 monoxygenases system (*6, 7*). Indeed, CPR has been suggested as a potential source of endogenous oxidative stress and DNA damage (*8*). Hence, CPR inhibition may reduce ROS generation and oxidative stress in different diseases of the liver.

In mammals, the expression of liver CPR is regulated by thyroid hormone (*9*−*11*). It has been reported that depletion of thyroid hormone by hypophysectomy (*9*) or treatment with the antithyroid drugs methimazole (*9*) or propylthiouracil (*11*) significantly reduces CPR activity and protein in the liver, an effect that is reversed by thyroxine therapy. However, manipulation of thyroid hormone levels is considered a nonspecific method of CPR inhibition because it affects a number of other physiological processes. Additionally, chemical inhibitors of CPR, such as diphenyleneiodonium (*12*) and α-lipoic acid (*13*), usually exert other nonspecific effects. On the other hand, inhibition of protein expression using antisense oligos or by RNA interference could potentially be relatively specific (*14*). Therefore, we chose to use the latter approach in our studies by applying a phosphorodiamidate morpholino oligo (PMO) (*15*) against CPR. PMOs are among the modified DNA analogues that block the translation initiation through an RNase-H independent process (*15, 16*). The modification of DNA in PMOs include replacement of deoxyribose sugars by morpholine and the charged phosphodiester linkages by nonionic phosphorodiamidate linkages (*15*). Consequently, PMOs are neutral and resistant to degradation by nucleases. Additionally, in contrast to phosphorothioates, which are known to bind nonspecifically to extracellular, cell surface, and intracellular proteins, PMOs exhibit little or no nonspecific protein binding (*15, 16*).

Because of their large size and physicochemical properties, unassisted delivery of antisense oligos into the cells is usually very limited after both in vitro and in vivo applications (*17*−*20*). Consequently, different methods, such as viral and nonviral vectors, have been used to improve their cellular delivery and effects. One of these methods is the use of avidin as a cationic carrier for delivery of biotinylated oligos (*21*−*24*). This method is based on the high-affinity binding of avidin to biotin (*25*) and entry of the avidin-bound cargo to the cell by endocytosis (*21*). Indeed, previous studies showed that the avidin−biotin approach can deliver antisense oligos or peptides to the isolated bovine capillaries in vitro (*21*) or antisense oligos to the liver of rodents in vivo (*23, 24*). However, we are not aware of any studies demonstrating that such an approach can indeed result in gene knockdown. Therefore, our main aim for the current study was to investigate the effectiveness of the avidin−biotin approach for delivery of a PMO antisense against CPR to a rat liver cell model. Because the binding of the avidin−biotinylated antisense to the target mRNA may be affected by the linker between biotin and antisense, we also tested the effects of linker length and cleavability on the effectiveness of the conjugates. Finally, the effectiveness of such an approach was tested in a

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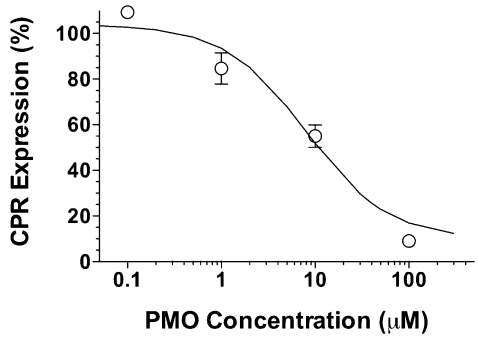

Figure 1. Relationship between the concentrations of unmodified PMO and CPR protein expression in rat liver epithelial cells. Cells were incubated with different concentrations of PMO in the presence of a 2 μM concentration of the transfection reagent Endoporter for 48 h. CPR expression was determined in the cell lysate by Western blot analysis. Symbols and bars represent experimental mean and SD ($n = 3$), respectively, and the line represents the model fitted relationship.

preliminary in vivo study in rats. Details of the experimental procedures are available in the Supporting Information.

Rat liver epithelial (WB-F344) cells (*26, 27*) were incubated for 48 h in the presence of 0.0, 0.1, 1, 10, and 100 μM concentrations of a 25-mer phosphorodiamidate morpholino oligo (PMO) against CPR and 2 μM of an in vitro transfection reagent (Endoporter, EP). Afterward, cells were lysed, and the lysate was used for CPR protein analysis using Western blotting method. Unmodified PMO showed dose-dependent decreases in CPR protein expression (Figure 1). Whereas a 0.1 μM concentration of PMO showed no inhibition of CPR, a 100 μM concentration inhibited CPR protein expression by ~90%. The nonlinear regression fit to the data revealed an IC_{50} value of 8.05 μM and a maximum inhibition of 94.0% for the unmodified PMO in the presence of EP transfection reagent.

Next, four different linkers (L1, L2, L3, and L4; Figure 2) were used to biotinylate the PMO against CPR. The biotinylated PMOs were characterized before and after retention of the biotinylated moiety on an avidin affinity column. The pre- and post-avidin column samples were then subjected to size exclusion HPLC to quantitate the degree of biotinylation. Figure 3 shows the chromatograms of the biotinylated PMO with the L1 linker before (Figure 3A) and after (Figure 3B) passage through the avidin column. The difference between the post-column and precolumn peak areas reflects the biotinylated fraction retained on the avidin column. The percentages of biotinylation, estimated from the postcolumn and precolumn samples, analyzed in triplicate, were 80.8, 74.9, 86.6, and 87.9 for the samples with L1, L2, L3, and L4 linkers, respectively.

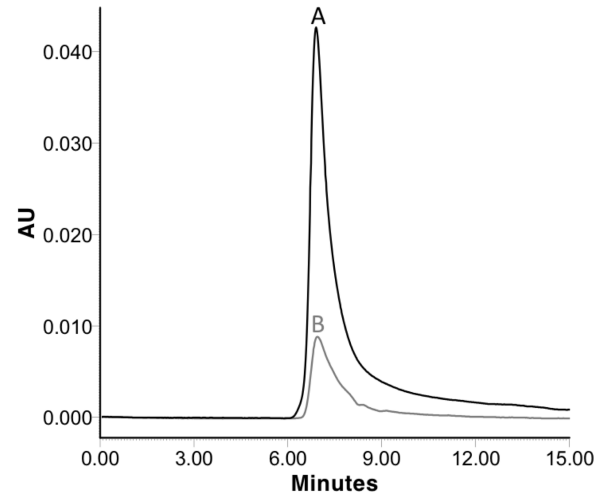

Figure 3. Representative chromatograms of a biotinylated PMO with linker 1 before (A) and after (B) passage through an avidin column that retains the biotinylated PMO. Chromatograms A and B represent the total (biotinylated plus free PMO) and free PMO, respectively.

These data indicate a relatively high degree of biotinylation with relatively small differences among biotinylation reagents with different linkers.

To determine the effect of biotinylation of PMO on its antisense effect, a 5 μM concentration of PMO biotinylated with L1 (PMO-L1-Biotin), L2 (PMO-L2-Biotin), L3 (PMO-L3-Biotin), or L4 (PMO-L4-Biotin) was incubated with WB cells for 48 h in the presence of EP. Additionally, unmodified PMO and invert PMO were used for comparison. As demonstrated in Figure 4, all four biotinylated PMOs inhibited CPR protein expression at least as effectively as the unmodified PMO (Figure 4). Interestingly, the biotinylated PMOs with the L1 (PMO-L1-Biotin) and L4 (PMO-L4-Biotin) linkers showed significantly (P <0.01 and <0.05, respectively) higher inhibition of CPR expression, compared with the unmodified PMO (Figure 4). As expected, the invert PMO did not significantly affect the CPR protein expression.

To determine the effectiveness of avidin for delivery of the biotinylated PMO, biotinylated PMOs were conjugated with avidin at a 1:1 molar ratio. The avidin conjugated biotinylated PMOs (5 μM) with L1 (PMO-L1-Biotin:Avidin), L2 (PMO-L2-Biotin:Avidin), L3 (PMO-L3-Biotin:Avidin), or L4 (PMO-L4-Biotin:Avidin) linkers were then incubated with WB cells for 48 h in the absence of the transfection reagent EP. Whereas avidin-conjugated biotinylated PMO with the short linker (L1) did not show any inhibitory effect, the two conjugates with the

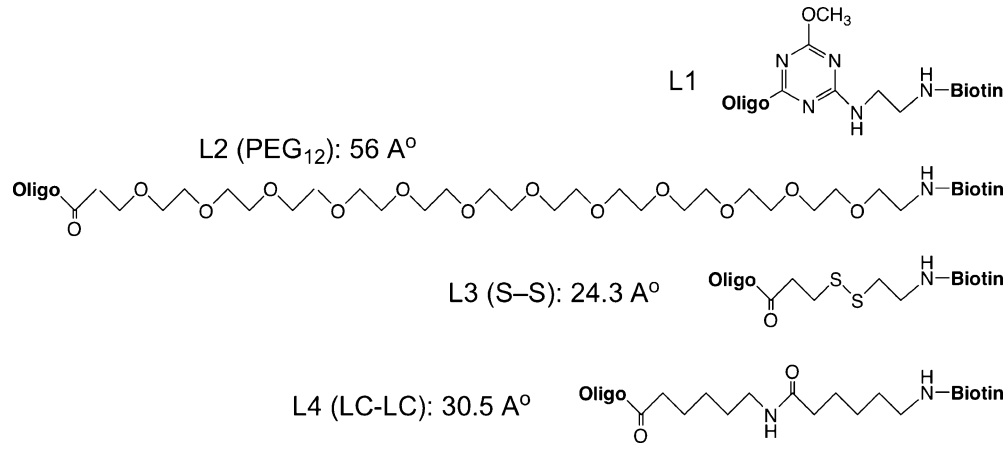

Figure 2. Chemical structures of various biotinylated linkers used in this study.

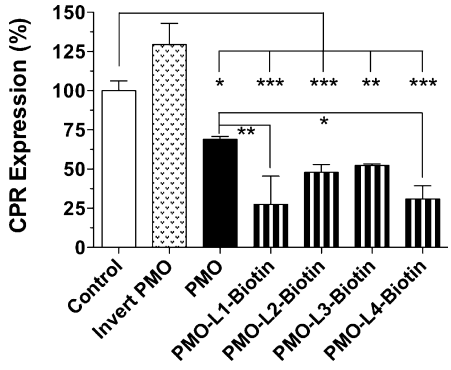

Figure 4. Effects of biotinylation with reagents containing linkers 1−4 on the inhibition of CPR protein expression by PMO in rat liver epithelial cells. Cells were incubated with a 5 μM concentration of PMO in the presence of a 2 μM concentration of the transfection reagent Endoporter for 48 h. CPR expression was determined in the cell lysate by Western blot analysis. Columns and bars represent mean and SD (n = 3), respectively. *P < 0.05, **P < 0.01, and ***P < 0.001 (one-way ANOVA, followed by Tukey's post hoc analysis).

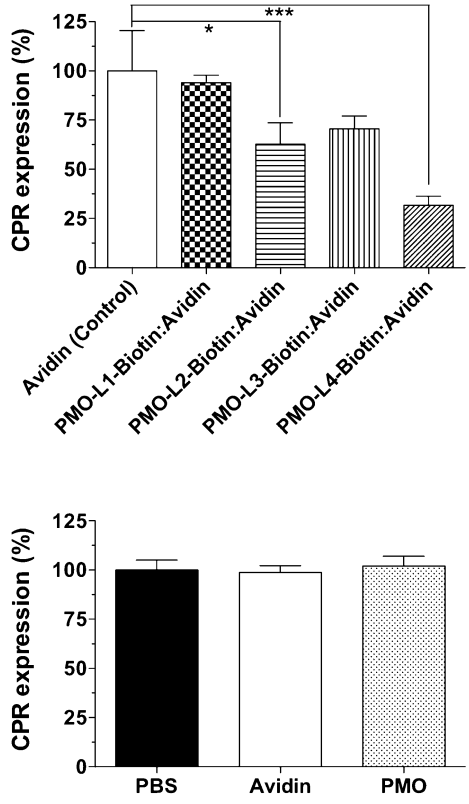

Figure 5. Effects of avidin-mediated delivery of PMO biotinylated with reagents containing linkers 1−4 on the inhibition of CPR protein expression in rat liver epithelial cells. Cells were incubated with a 5 μM concentration of avidin alone (control) or avidin−biotin PMOs (top) or with phosphate buffered saline (PBS) or a 5 μM concentration of avidin alone or PMO alone (bottom) in the absence of the transfection reagent Endoporter for 48 h. CPR expression was determined in the cell lysate by Western blot analysis. Columns and bars represent mean and SD (n = 3), respectively. *P < 0.05 and ***P < 0.001 (one-way ANOVA, followed by Tukey's post hoc analysis).

long linkers (L2 and L4) showed significant reductions in CPR protein expression (P < 0.05 and P < 0.001, respectively) (Figure 5, top). As for the conjugate with the cleavable linker (L3), the reduction in the CPR protein expression did not reach statistical significance. Overall, among all the tested conjugates, the avidin-conjugated biotinylated PMO with L4 showed the highest degree of CPR protein inhibition (~70%). Control experiments with

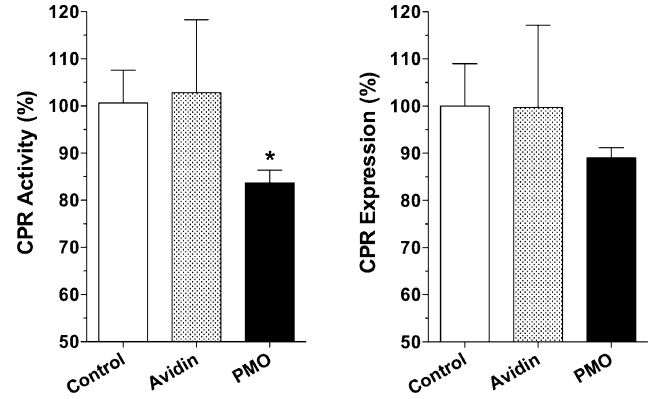

Figure 6. Microsomal CPR activity (left) and expression (right) in rat livers after intravenous administration of vehicle (control), 40 mg/kg/day of avidin (Avidin), or 5 mg/kg/day of PMO-L4-Biotin:Avidin (PMO) for 3 days. Microsomal CPR expression and activity were determined 24 h after the last dose. Columns and bars represent mean and S.D., respectively (n = 8 for the control group and 4 for the avidin and PMO groups). * Significant difference (P < 0.05, one-way ANOVA, followed by Tukey's post hoc analysis) between PMO and control and between PMO and avidin.

phosphate-buffered saline (PBS), avidin alone, and PMO alone demonstrated that neither PMO alone in the absence of any transfection reagent nor avidin alone had any suppressive effects on CPR expression (Figure 5, bottom).

Finally, in a preliminary study, male Sprague−Dawley rats (75−100 g) were treated intravenously with vehicle, avidin (40 mg/kg), or 5 mg/kg/day doses of PMO-L4-Biotin:Avidin for 3 days and the microsomal CPR activity (*28*) and protein expression were determined. The avidin-conjugated PMO showed (Figure 6) modest, but significant, reductions in the rat liver microsomal CPR activity, relative to the control (17% decline) and Avidin (19% decline) groups. As for the CPR protein contents, an 11% reduction in the PMO group, compared with the control or Avidin group, did not reach statistical significance using multiple comparisons.

One of the main hurdles in the CPR research is that there are no specific chemical inhibitors for this protein to be used for delineation of its role in oxidative injury. Hence, we sought to use the antisense approach in this study to specifically inhibit the translation of CPR at its mRNA level. Our dose−response data (Figure 1) using the PMO against CPR showed a dose-dependent inhibition of CPR in WB cells with an IC_{50} of 8 μM and 90% inhibition of CPR at a concentration of 100 μM (Figure 1). However, the reported (*29*) turnover half-life of CPR in rat liver microsomes is 29−35 h, which suggests that a complete inhibition of protein expression is expected to result in a maximum of 70% decline in the protein levels after 48 h of incubation. Therefore, a higher degree of inhibition observed after the PMO concentration of 100 μM (~90%, Figure 1) suggests that the PMO antisense used in our study may exert some nonspecific effects at this high concentration.

To use avidin as a carrier, the antisense oligos are first biotinylated to take advantage of the high-affinity binding of avidin to biotin, which has a dissociation half-life of 89 days (*25*). Because biotinylation of antisense oligos may alter their physicochemical properties and binding to the target mRNA, we studied the effects of biotinylation with different linkers on the CPR inhibitory effect of our PMO antisense. Biotinylation of PMO not only did not adversely affect the inhibition of CPR expression, but also caused an improvement of their effects in the case of PMO-L1-Biotin and PMO-L4-Biotin (Figure 4). The reasons for this improvement are not clear from our studies. However, possible explanations could be an improvement in

the EP-mediated delivery of the PMO antisense to the cytosol and/or improved binding of the PMO to the target mRNA as a result of biotinylation. These results are in agreement with those reported by Boado and Pardridge (*22*) who showed that RNase H caused degradation of *tat* sense RNA in the presence of a biotinylated phosphodiester antisense oligo against *tat*. However, they did not compare the efficacy of the biotinylated oligo with that of nonbiotinylated oligo. Nevertheless, our studies appear to be the first examination of the effects of biotinylation on the inhibitory activity of PMO oligos.

An in vitro study using isolated bovine capillaries (*21*) showed that avidin acts as a carrier for cellular uptake of a biotinylated antisense oligonucleotide or a model peptide. It was suggested that the avidin-mediated uptake is associated with endocytosis and is based on the cationic nature of the avidin protein. Additionally, a more recent study (*24*) showed that a radiolabeled biotinylated oligo conjugated to avidin was internalized into the mouse hepatocytes without the assistance of transfection reagents. However, neither of these studies reported the effects of the antisense or peptide. When we first tested the inhibitory effects of an avidin conjugate of the commercially available biotinylated PMO against CPR (PMO-L1-Biotin:Avidin) in our WB cells, we were disappointed by the lack of inhibitory effect of this conjugate (Figure 5). Because the linker between biotin and PMO was short (L1, Figure 2), we hypothesized that this lack of effect is due to the steric hindrance of avidin, impeding the interaction of the oligo with its target mRNA. Therefore, in a subsequent study, we biotinylated our PMO with reagents that contained long (L2 and L4) or cleavable (−S−S−) linkers (Figure 2). In agreement with our hypothesis, all these linkers inhibited the CPR expression, although the reduction did not reach statistical significance with the cleavable linker (L3; Figure 5). Interestingly, CPR inhibition with the avidin-mediated delivery of PMO with L2 or L4 linkers (Figure 5) was as effective as or more effective than the inhibitory effect observed with the EP-mediated delivery of the unmodified PMO (Figure 4). These results suggest that avidin-mediated delivery using appropriate linkers may be an alternative approach to transfection reagents in the intracellular delivery of PMOs in vitro.

Although the in vivo distribution of avidin conjugates of biotinylated oligos has been reported previously in rodents (*23, 24*), we are not aware of any in vivo studies testing the inhibitory effects of these conjugates. The previously reported distribution studies (*23, 24*) suggested high accumulation of the conjugates in the liver. Therefore, we tested the efficacy of PMO-L4-Biotin:Avidin, which had shown the strongest in vitro effect (Figure 5), in a preliminary study in rats. The in vivo study (Figure 6) showed for the first time that an avidin-mediated in vivo gene knockdown is feasible. However, the relatively low level of inhibitory response suggests that such an approach may be limited by the potency of the gene knockdown agent. This is because, to deliver a 5 mg/kg dose of CPR PMO (M_w of 8476), we had to administer a 40 mg/kg dose of avidin (M_w of 68000) in a 1:1 molar ratio used in our studies. This is a relatively high dose for in vivo administration of avidin, limiting the dose of oligo that may be delivered by this approach. Thus, the avidin-mediated in vivo gene knockdown approach may be more suitable for delivery of more potent oligos or other gene knockdown agents, such as siRNAs, that inhibit CPR expression at nanomolar concentrations (*30*).

In conclusion, our studies show for the first time that avidin-mediated intracellular delivery of biotinylated oligos can effectively knock down target genes in vitro. Further, it was shown that the efficacy of an avidin-conjugate of biotinylated PMO antisense against rat liver epithelial cell CPR depends on the length of the linker between the biotin and PMO, with longer linkers being more effective than a short linker. Although preliminary studies indicated feasibility of in vivo gene knockdown by the avidin−biotin approach, the method may be limited by the requirement for relatively large amounts of avidin. Further studies are needed to test this approach for in vivo delivery of more potent agents, which require smaller amounts of avidin administration.

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Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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