

Hairpin Ribozyme Genes Curtail Alcohol Drinking: from Rational Design to *in vivo* Effects in the Rat

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Ribozyme genes were designed to reduce voluntary alcohol drinking in a rat model of alcohol dependence. Acetaldehyde generated from alcohol in the liver is metabolized by the mitochondrial aldehyde dehydrogenase (ALDH2) such that diminishing ALDH2 activity leads to the aversive effects of blood acetaldehyde upon alcohol intake. A stepwise approach was followed to design genes encoding ribozymes targeted to the rat ALDH2 mRNA. *In vitro* studies of accessibility to oligonucleotides identified suitable target sites in the mRNA, one of which fulfilled hammerhead and hairpin ribozyme requirements (CGGUC). Ribozyme genes delivered in plasmid constructs were tested in rat cells in culture. While the hairpin ribozyme reduced ALDH2 activity 56% by cleavage and blockade ($P < 0.0001$), the hammerhead ribozyme elicited minor effects by blockade. The hairpin ribozyme was tested *in vivo* by adenoviral gene delivery to UChB alcohol drinker rats. Ethanol intake was curtailed 47% for 34 days ($P < 0.0001$), while blood acetaldehyde more than doubled upon ethanol administration and ALDH2 activity dropped 25% in liver homogenates, not affecting other ALDH isoforms. Thus, hairpin ribozymes targeted to 16 nt in the ALDH2 mRNA provide durable and specific effects *in vivo*, representing an improvement on previous work and encouraging development of gene therapy for alcoholism.

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Subject Category: Aptamers, ribozymes and DNAzymes; Therapeutic proof-of-concept

Introduction

The hammerhead and hairpin ribozymes were initially identified as functional RNA entities embedded in the concatemeric form of virusoids or virus satellites known to contribute to plant diseases, but they were rapidly tailored into autonomous molecules with therapeutic potential for human diseases.^{1,2} Although virusoid ribozymes perform an autologous (in *cis*) cleavage which has been incorporated into synthetic riboswitches with biomedical purposes,³ most applications in gene therapy exploit cleavage in *trans* to tune down the expression of a transcript, typically a mature messenger RNA. Like several other molecular tools, ribozymes accomplish knockdown of RNA based on antisense recognition of the target. However, while antisense oligonucleotides, siRNA and miRNA rely mostly on cellular functions to exert their silencing effects,^{4,5} hammerhead and hairpin ribozymes have the advantage of eliciting self-supported effects.

Some notable examples of the development of ribozymes with pharmacological purposes are those for infections by the human immunodeficiency virus and the hepatitis C virus, and for various forms of cancer.⁶ Anti human immunodeficiency virus efforts, recently reviewed,⁷ have reached clinical trials in several instances. However, multifactorial diseases such as alcohol use disorders may also be addressed with ribozymes.

The susceptibility to develop alcoholism is determined in great part (~60%) by genetic factors⁸ but virtually full protection against alcohol abuse and alcoholism can be conferred by a single gene: individuals carrying a point mutation in the

aldehyde dehydrogenase 2 (ALDH2) gene (allele *ALDH2*2*) have a reduced ability to metabolize the acetaldehyde generated from ethanol in the liver by alcohol dehydrogenase (ADH) and also have a very low incidence of alcoholism. Acetaldehyde is metabolized into acetate in liver mitochondria by ALDH2, a homotetrameric enzyme which, through a dominant negative effect,⁹ is rendered inactive by a Glu-497Lys mutation. Acetaldehyde accumulates in *ALDH2*2* carriers who consume ethanol and results in a marked protection against alcoholism ranging from 66–75% in heterozygotes to 100% in homozygotes.¹⁰ Acetaldehyde has dual counteracting actions: an aversive effect in the periphery and a reinforcing effect in the brain.¹¹ Although acetaldehyde does not cross the blood brain barrier, it may also be generated from ethanol in the brain, but mainly by catalase, serving as a reinforcing prodrug which generates salsolinol upon condensation with dopamine.

A pharmacologic protection similar to that conferred by the *ALDH2*2* allele may be achieved by chemical inactivation of ALDH2 with disulfiram, the first medication available for the treatment of alcohol use disorders and widely prescribed today. While naltrexone and acamprosate are also currently available,¹² they are less effective than disulfiram and aim to modulate the neuronal mechanisms of alcohol consumption. Disulfiram is a prodrug requiring supervised daily intake to be effective,¹³ and has toxic effects due to nonspecific actions.¹⁴ Gene therapy may circumvent both problems by affording a prolonged inhibition of the ALDH2 mRNA in a very specific manner.

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Gene silencing strategies to curtail alcohol drinking have been tested in rats. The use of antisense oligonucleotides targeted to the ALDH2 mRNA in the Lewis strain provided proof of concept,¹⁵ while the use of an adenoviral vector to deliver an ALDH2 antisense gene to *Universidad de Chile-Bibulous* (UChB) rats, a well-established rodent model of voluntary ethanol consumption,¹⁶ showed it is possible to obtain a marked and durable reduction on drinking by genetic means.¹⁷ In this work, a stepwise approach was followed to design ribozyme genes capable of supporting knockdown of the ALDH2 mRNA with the aim of developing a more specific gene therapy for alcoholism based on the aversive effect of acetaldehyde upon alcohol intake. First, accessible sites in the rat ALDH2 mRNA were identified *in vitro*. Three target sites were chosen for the design of hammerhead ribozymes that were tested *in vitro* and in cell culture by delivering ribozyme genes in plasmid vectors. One of the targets allowed the design of a hairpin ribozyme that was tested in cell culture in two different gene constructs. The best assembly was tested *in vivo* by adenoviral delivery to high alcohol drinker rats to assess the ability of the hairpin ribozyme to curtail voluntary alcohol drinking.

Results

Accessibility of the rat ALDH2 mRNA *in vitro*

The preferred NUH triplets in an RNA target for cleavage by a hammerhead ribozyme are GUC, CUC, and UUC,¹⁸ of which the rat ALDH2 mRNA has 78. The accessibility of 30 of these candidate sites for ribozyme design, or of their immediate vicinity (± 10 nt), was tested with 16 DNA oligonucleotides (18–23 nt in length) in an RNase H protection assay; oligonucleotides were allowed to bind individually to the folded ALDH2 mRNA at an RNA:DNA ratio of 1:25 (1:100 and 1:250 gave similar results in preliminary assays). Four regions of the Sprague–Dawley rat ALDH2 mRNA were found to be fully accessible in the folded RNA (not protected from RNase H): nucleotides 510–529, 576–597, 1478–1500, and 1534–1552. Results are shown in **Figure 1**.

Design of anti-*Aldh2* hammerhead ribozymes

Additional demands were imposed on accessible mRNA areas to be considered suitable targets for hammerhead ribozymes: lack of intra and inter molecular structures between the arms flanking the cleavage site, intermediate GC content to allow both reasonable binding to and dissociation from the ribozyme, and uniqueness of sequence as determined by comparison to all rat mRNAs by BLAST,¹⁹ particularly to other isoforms of ALDH. Three hammerhead ribozymes (**Figure 2**) were designed according to standard guidelines²⁰: the catalytic core of the (+)sTRSV (positive strand of the tobacco ringspot virus satellite)^{21,22} and arms of 8–10 nucleotides to bind to the substrate on either side of the unpaired H nucleotide. Ribozymes were named RzUUC9, RzGUC19, and RzGUC20 to specify triplet type and position in the target (*e.g.*, RzGUC20 targets the 20th GUC triplet of the mRNA) and were cloned in pGEM-3Zf(+) for synthesis by *in vitro* transcription. Counterpart ribozyme constructs in which A9 is replaced by a C to abolish cleavage were also made with the aim of dissecting silencing activity into blockade and cleavage components.

Cleavage activity of hammerhead ribozymes *in vitro*

The ALDH2 mRNA (1939 nts) was incubated with each ribozyme at 37°C for 16 hours. Several magnesium ion concentrations were tested (1, 5, 10, and 25 mmol/l), 10 mmol/l providing the best cleavage; Na⁺ (10 mmol/l) and K⁺ (140 mmol/l) improved the reaction. Ribozyme:mRNA molar ratios of 1:10 and 1:5 did not afford detectable cleavage whereas 1:1 and 5:1 ratios yielded increasing amounts of cleavage products. Ribozymes RzUUC9 and RzGUC20 were able to cut the substrate, as evidenced by the appearance of RNA products of 568 and 348 nt respectively (**Figure 2**), but RzGUC19 was not. As expected, the three control ribozymes were unable to cut the target RNA.

Gene silencing activity of hammerhead ribozyme genes in rat cells in culture

Ribozyme coding sequences were cloned downstream of the CMV promoter in plasmid pAC-CMV to ascertain ribozyme activity in a rat hepatoma cell line (H4-II-E-C3) in which the ALDH2 mRNA sequence is the same as that of the Sprague–Dawley rat.¹⁵ Cells were lipofected with plasmid constructs encoding RzUUC9, RzGUC19 or RzGUC20, or their control ribozymes. Control transfections were carried out with the parent plasmid or the transfection agent alone. ALDH2 activity was measured in cell lysates of cultures harvested 48 hours after lipofection, time at which ~25% of the initial activity remains given that the ALDH2 half-life is 22 hours in these cells.¹⁵

Plasmids encoding RzUUC9, RzGUC19, and their control ribozymes resulted in minor decreases (8–10%) in ALDH2 activity (data not shown), while those encoding RzGUC20 and its control doubled this effect, affording reductions of 15.8% and 24.1% respectively (**Figure 3**). Differences between wild type ribozymes and their control ribozymes were not significant ($P > 0.05$), indicating that their effects were attained through blockade of the target, *i.e.* an antisense mechanism only (no cleavage). These data suggested that the target site for RzGUC20 (nts 1551–1571) is indeed accessible to a ribozyme in cells in culture, allowing the possibility that a hairpin ribozyme targeted to that same site might have a greater effect on silencing the ALDH2 mRNA.

Design of anti-*Aldh2* hairpin ribozymes and gene constructs

The hairpin ribozyme derived from (–)sTRSV, the negative strand of the tobacco ringspot virus satellite, requires a BNGUC sequence in the target for cleavage of the phosphodiester bond preceding the GUC triplet. The cleavage site for the hammerhead RzGUC20 ribozyme is in the context of a CGGUC pentanucleotide, which fulfills the hairpin ribozyme requirement, thus allowing a direct comparison between hammerhead and hairpin ribozyme effects on the same target. A hairpin ribozyme named RzCG20 (CG being the BN dinucleotide preceding the 20th GUC triplet of the mRNA), and a control ribozyme (cRzCG20) —in which G8 is replaced by a C to abolish cleavage— were designed according to standard criteria²³ to bind to nts 1554–1569 of the rat ALDH2 mRNA (**Figure 4**). These ribozyme coding sequences were cloned in pGEM-3Zf(+) for synthesis by *in vitro* run-off transcription in order to confirm that RzCG20 cleaves de ALDH2 mRNA *in vitro* while cRzCG20 does not

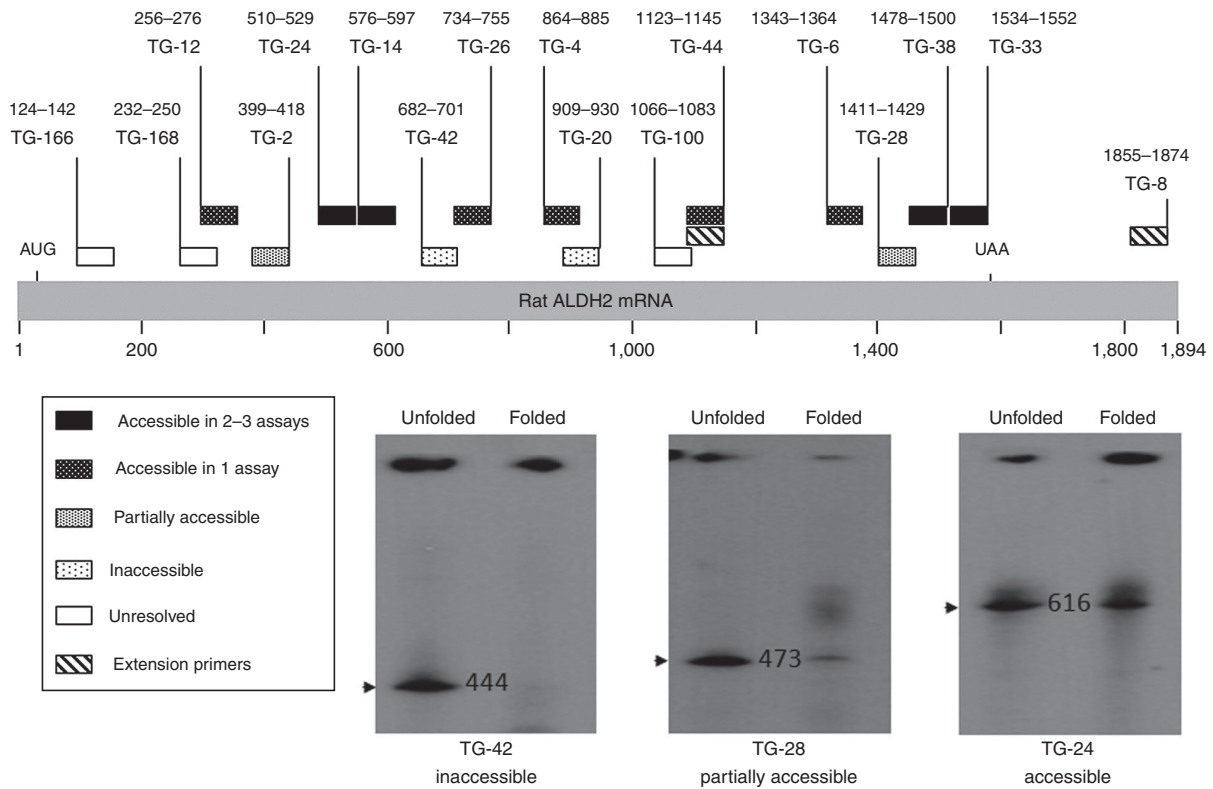


Figure 1 Accessibility of the rat aldehyde dehydrogenase 2 (ALDH2) mRNA to oligonucleotides *in vitro*. The rat ALDH2 mRNA was synthesized by *in vitro* transcription, renatured, and subjected to RNase H digestion after binding of each of the 16 DNA oligonucleotides shown (TG-#, nonstriped boxes). Accessibility of the target regions in the folded mRNA (nucleotide positions specified above each oligonucleotide) was determined by extension of 5'-radiolabeled primers (TG-8 or TG-44; striped boxes) with reverse transcriptase followed by electrophoresis in 5% polyacrylamide denaturing gels and autoradiography. Positive controls in which each oligonucleotide was allowed to bind to the unfolded mRNA were run in parallel. Representative examples of gel data are shown for three oligonucleotides yielding different outcomes. The size of the resulting cDNA is indicated in nucleotides. Experiments were performed at least three times for each oligonucleotide. Results were classified in five categories indicated by the pattern of the boxes representing the oligonucleotides: unresolved (white boxes), inaccessible, partially accessible, accessible in one assay, or accessible in 2–3 assays (black boxes).

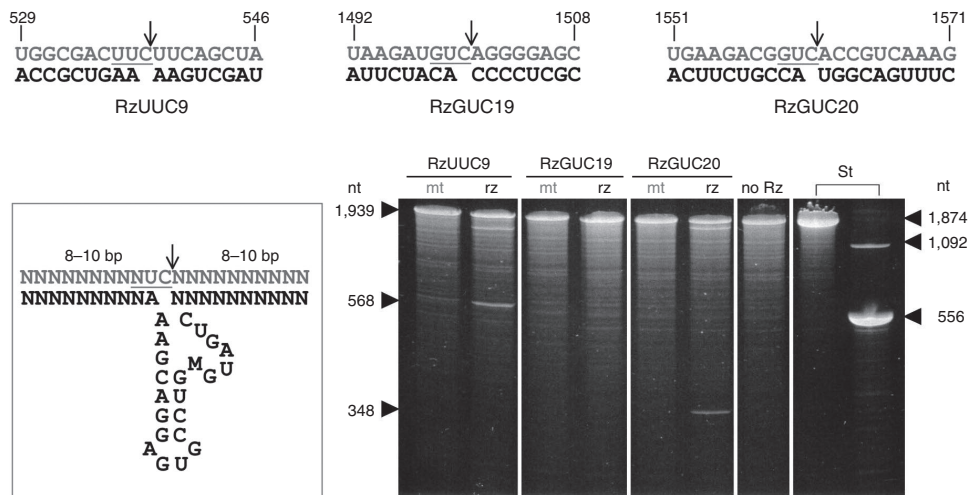


Figure 2 Design of anti-*Aldh2* hammerhead ribozymes and their cleavage activity *in vitro*. Three hammerhead ribozymes and their control mutant ribozymes were designed according to the (+)sTRSV ribozyme (boxed); the identity of M is A in the wild type sequence ribozymes (rz) and C in the mutant ribozymes (mt). The sequence of the arms of each ribozyme bound to its target region in the aldehyde dehydrogenase 2 (ALDH2) mRNA is shown; nucleotide positions in the target are specified. The arrows indicate the sites of expected cleavage. Ribozymes and the target mRNA (1939 nt) were synthesized by run-off *in vitro* transcription of the pGEM-3Zf(+) ribozyme constructs or the pGEM-T Easy mRNA construct linearized with *Hind*III or *Sal*I respectively, and cleavage capacity was tested at a 5:1 ratio overnight at 37°C. Results are shown in a denaturing 5% polyacrylamide gel stained with ethidium bromide. Size standards are: ribosomal RNA of H4-II-E-C3 cells (1875 nt) and the transcript from the plasmid encoding the mRNA linearized with *Sma*I or *Cl*aI (1092 and 556 nt respectively).

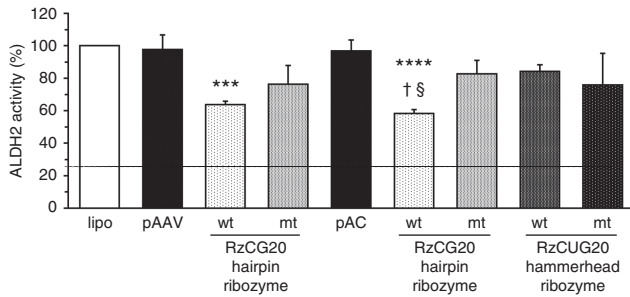


Figure 3 Aldehyde dehydrogenase 2 (ALDH2) mRNA silencing activity of ribozyme genes in rat cells in culture. Rat hepatoma H4-II-E-C3 cells were lipofected with plasmids encoding the three hammerhead ribozymes, the hairpin ribozyme, and their control ribozymes (cleavage activity disabled); shown are the data for the RzGUC20 hammerhead ribozyme encoded in the pAC-CMV plasmid (pAC), and for the RzCG20 hairpin ribozyme encoded in the pAC-CMV plasmid or the pAAV-CMV plasmid (pAAV). The aldehyde dehydrogenase 2 (ALDH2) enzymatic activity was measured 48 hours later. Control transfections were performed with each plasmid vector (pAC-CMV or pAAV-CMV) or no DNA (the cationic lipid agent alone), identified as pAC, pAAV, and lipo respectively. Four experiments were performed on different days ($n = 4$), each in quadruplicate; enzymatic activity was measured in duplicate and expressed as percent of the activity of untreated cells (mean \pm SD). Statistical significance was obtained by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test with a 95% confidence interval (CI). Effects given by the hairpin wild type ribozyme constructs (RzCG20) are significantly different from those given by the parent plasmids (**** $P < 0.0001$ and *** $P < 0.001$). When in the pAC-CMV construct, RzCG20 also shows significant differences from its control ribozyme (cRzCG20) construct ($\dagger P < 0.05$) and from the hammerhead RzGUC20 construct ($\S P < 0.01$). The line drawn at 25% indicates the enzymatic activity expected at 48 hours of culture for complete ALDH2 mRNA knockdown.

(shown in **Figure 4**). The ribozyme coding sequences were also cloned downstream of the CMV promoter in two different plasmids, pAC-CMV and pAAV-CMV, with the purpose of comparing the effects of molecular context (in *cis*) on hairpin ribozyme action in cells in culture.

Gene silencing activity of hairpin ribozyme genes in rat cells in culture

Plasmids encoding the RzCG20 and cRzCG20 hairpin ribozymes afforded greater reductions in the ALDH2 activity of lysates from cultures of transfected rat hepatoma cells than plasmids encoding hammerhead ribozymes (**Figure 3**). When cloned in pAC-CMV, ribozymes RzCG20 and cRzCG20 afforded reductions of 41.8% and 17.3% respectively and, when cloned in pAAV-CMV, ALDH2 activity dropped 36.3% and 23.7%. The difference between RzCG20 and its control ribozyme in pAC-CMV ($P < 0.05$) indicated that silencing occurred by both cleavage and blockade. The cleavage contribution may be estimated by subtracting the effect of the control ribozyme construct (the blockade component) from that of the RzCG20 construct. The effect of RzCG20 in the pAC-CMV construct is given by 58.5% cleavage and 41.5% blockade, while in the pAAV-CMV construct it is given by 34.7% and 65.3% respectively. Although the silencing effects of RzCG20 are not significantly different between molecular contexts ($P > 0.05$), the length of the transcripts and the location of the ribozyme in them are quite different (inset **Figure 4**). The largest effect observed—42% reduction in ALDH2 activity for RzCG20 in pAC-CMV— amounts to 56% when taking into account the protein half-life and that the cells were harvested 48 hours after lipofection, anticipating that this hairpin ribozyme gene might perform well *in vivo*.

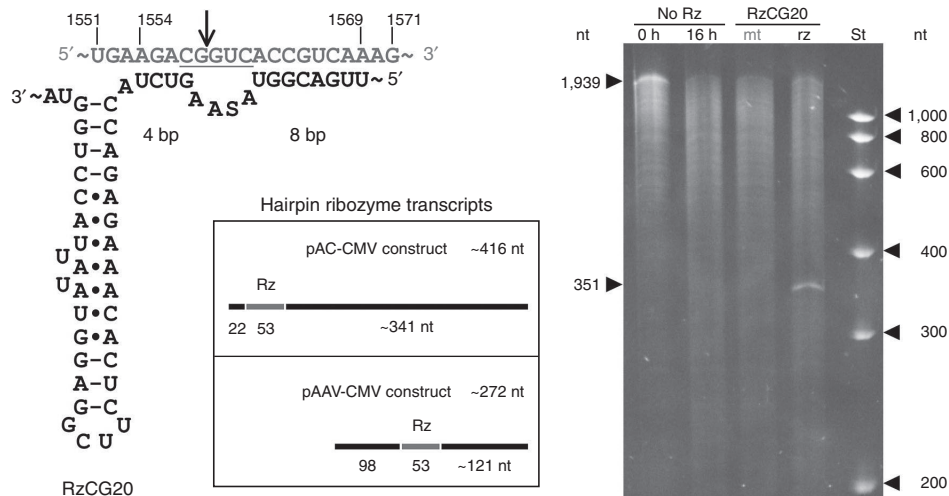


Figure 4 Design of anti-*Aldh2* hairpin ribozymes and their cleavage activity *in vitro*. A hairpin ribozyme (RzCG20) and its control mutant ribozyme were designed according to the (-)sTRSV ribozyme. The sequence of both ribozymes bound to the target region in the aldehyde dehydrogenase 2 (ALDH2) mRNA is shown; the identity of S is G in the wild type sequence ribozyme and C in the mutant ribozyme. Nucleotide positions in the target are specified and the arrow indicates the site of expected cleavage. Ribozymes and the target mRNA (1939 nt) were synthesized by run-off *in vitro* transcription of the pGEM-3Zf(+) ribozyme constructs or the pGEM-T Easy mRNA construct linearized with *HindIII* or *SalI* respectively, and cleavage capacity was tested at a 5:1 ratio overnight at 37°C. Results are shown in a denaturing 5% polyacrylamide gel stained with ethidium bromide. Size standard: RiboRuler Low Range RNA Ladder (Fermentas). The inset shows the hairpin ribozyme transcripts expected in rat cells in culture from the two types of plasmid constructs made for this purpose and used to transfect H4-II-E-C3 cells. The transcriptional units in the pAC-CMV constructs for the wild type and the mutant hairpin ribozymes were transferred to the adenoviral vectors used for transduction of UChB rats to generate the ribozymes *in vivo*.

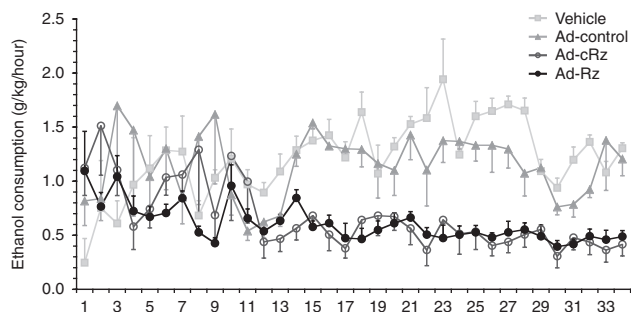


Figure 5 Voluntary alcohol intake of UChB rats transduced with adenoviral vectors encoding hairpin ribozymes. Inhibition of ethanol intake by UChB rats transduced with adenoviral vectors carrying hairpin ribozyme genes (Ad-Rz, Ad-cRz) is shown; the wild type RzCG20 ribozyme and its control cRzCG20 ribozyme (bearing a point mutation to abolish cleavage) are targeted to the ALDH2 mRNA. UChB rats were allowed unrestricted access to 10% (v/v) ethanol and water for 30 days, time at which their voluntary ethanol consumption was ~7 g ethanol/kg/day. Ethanol was withdrawn and animals received a single i.v. injection of 2×10^{12} vp/kg of Ad-Rz, Ad-cRz, or Ad-control (encoding a nonfunctional control RNA), or vehicle. After 4 days of ethanol withdrawal (shown as day 1), animals were allowed access to 10% (v/v) ethanol for only 1 hour each day, an experimental design that leads to the intake of intoxicating amounts of ethanol in a short time. Water was continuously available. Points represent daily ethanol intake during the 1 hour ethanol access period (mean \pm SEM of five animals for each ribozyme group and four animals for each control group). ANOVA: $P < 0.0001$ between Ad-Rz and Ad-control; and between Ad-cRz and Ad-control. There were no statistically significant differences between the Ad-Rz and Ad-cRz groups. ANOVA, one-way analysis of variance; SEM, standard error of the mean.

In vivo effects of hairpin ribozyme genes in UChB rats transduced with adenoviral vectors

Adenoviral vectors carrying the gene cassettes for the RzCG20 and cRzCG20 hairpin ribozymes in the pAC-CMV construct were used to study whether these hairpin ribozymes were active as gene silencing agents *in vivo*; a third vector encoding a nonfunctional RNA of similar size served as control adenovirus. UChB rats, which have been selectively bred from the Wistar strain for high ethanol intake on a voluntary basis, were allowed free access to ethanol (10% v/v) and water for 30 days. Average alcohol intake was 7.0 ± 0.2 g/kg/day. Groups of five animals received each of the two ribozyme genes while four rats received each of the control treatments, *i.e.*, the adenoviral vector encoding the nonfunctional RNA, and vehicle only. Ethanol was withdrawn and rats were administered a single i.v. injection of one of the three adenoviral vectors (2×10^{12} viral particles/kg) or vehicle. Four days later animals were allowed access to 10% ethanol for only 1 hour each day, while water was permanently available. Alcohol and water consumption, and weight, were monitored for 34 days; alcohol was withdrawn and biochemical determinations (blood acetaldehyde and hepatic ALDH2 activity) were performed.

Effects on voluntary alcohol intake. Results (Figure 5) show that the voluntary ethanol intake of UChB rats that received the adenoviral vector carrying the RzCG20 gene (Ad-Rz) did not diminish significantly during the first three days. Subsequently, intake decreased in a cyclic mode (days 1–14) characterized by two or three days of diminished intake followed by a 1-day increase;

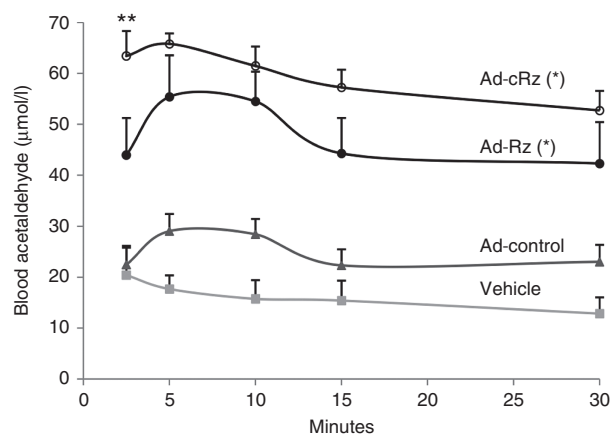


Figure 6 Blood acetaldehyde levels of UChB rats transduced with adenoviral vectors encoding hairpin ribozymes. Rats for which ethanol intake was measured during limited access to ethanol (Figure 5) were detoxified for at least one day (ethanol was withdrawn), and received a standard dose of ethanol (1 g/kg i.p.). Blood was sampled from the carotid artery at different times to determine acetaldehyde levels. Data are shown as the mean \pm SEM for each group. There are statistically significant differences between each point of both ribozyme groups (*) and the same time points for the Ad-control and vehicle groups (ANOVA $P < 0.001$). The acetaldehyde levels measured for the Ad-Rz and Ad-cRz groups were significantly different from each other (ANOVA $P < 0.0001$) only 2.5 minutes after ethanol injection (**). ANOVA, one-way analysis of variance; SEM, standard error of the mean; i.p., intraperitoneal.

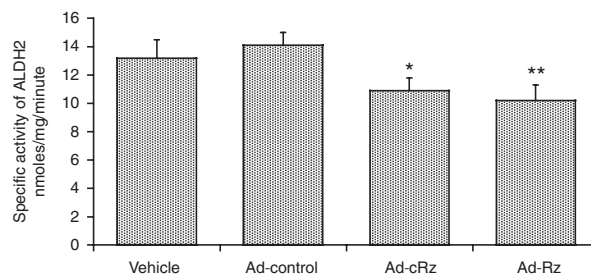


Figure 7 Liver aldehyde dehydrogenase 2 (ALDH2) activity in rats that received adenoviral vectors encoding hairpin ribozymes. UChB rats for which ethanol intake and blood acetaldehyde levels were measured ((Figure 5) and (6) respectively) were sacrificed and aldehyde dehydrogenase 2 (ALDH2) activities were determined in liver homogenates. Specific activity of ALDH2 shown as the mean \pm SEM of two independent measurements performed in duplicate. Statistically significant differences (Student's *t*-test) between the Ad-Rz and Ad-control groups (** $P < 0.01$), and between the Ad-cRz and Ad-control groups (* $P < 0.02$), are indicated. SEM, standard error of the mean.

intoxicating levels (>1 g/kg/hour) were only registered on day 10. In spite of this cyclic behavior the average intake in this period experienced a significant decrease ($P < 0.05$). In contrast to the first group, the animals receiving the control ribozyme vector (Ad-cRz) did not show significantly reduced drinking during days 1–14 of limited access to ethanol. Intoxicating amounts were ingested most days in a cyclic pattern characterized by a one-day decrease and 2–3 days of increase. During this first fortnight, the UChB rats that received the control adenovirus (Ad-control) did not show a reduction in their alcohol intake.

Following the first two weeks of restricted access to ethanol, rats treated with Ad-Rz or with Ad-cRz stabilized their ethanol intake until the experiment was ended (day 34). Taking into account days 15–34 only, rats treated with Ad-Rz and with Ad-cRz drank 0.51 ± 0.01 g/kg/hour and 0.50 ± 0.03 g/kg/hour respectively, which represent an inhibition of ethanol intake of ~58% when compared with that of rats treated with Ad-control (1.21 ± 0.50 g/kg/hour). If the entire period of 34 days of restricted access to ethanol is considered, alcohol intake of rats treated with Ad-Rz (0.61 ± 0.03 g/kg/hour) was slightly (although not significantly) lower than that of rats treated with Ad-cRz (0.67 ± 0.05 g/kg/hour), representing inhibitions of 47 and 42% respectively when compared with the 1.15 ± 0.50 g/kg/hour recorded for rats treated with Ad-control. Water consumption was not different between groups and weight increased normally throughout the experiment (data not shown).

Effects on blood acetaldehyde levels. Given that the administration of adenoviral vectors encoding the RzCG20 and the cRzCG20 hairpin ribozymes resulted in ~50% reduction in voluntary ethanol consumption in UChB rats, a relationship between curtailed drinking and increased acetaldehyde in blood was investigated. The animals for which ethanol intake was measured during limited access to ethanol were detoxified for at least one day (ethanol was withdrawn), a standard dose of ethanol (1 g/kg) was administered i.p., and blood was sampled from the carotid artery at different times to determine acetaldehyde levels by head space chromatography.

As shown in **Figure 6**, Ad-Rz generated a significant increase in arterial acetaldehyde, reaching a concentration of ~55 $\mu\text{mol/l}$, 5 minutes after ethanol administration, twice and more than three times that of animals treated with Ad-control (~29 $\mu\text{mol/l}$) or vehicle (~17 $\mu\text{mol/l}$) respectively. Likewise, also 5 minutes after ethanol administration, Ad-cRz resulted in a blood acetaldehyde concentration of ~65 $\mu\text{mol/l}$, more than twice and almost four times that of the animals injected with Ad-control or vehicle respectively. Although the control ribozyme appears to generate a greater increase in blood acetaldehyde than the ribozyme, the differences are not significant ($P > 0.05$), with the exception of the measurement at 2.5 minutes ($P < 0.0001$). Similarly, the apparent difference in the acetaldehyde levels between the control adenovirus and vehicle groups is not significant at any time point ($P > 0.05$). These measurements suggest that the reduction in alcohol intake in rats transduced with hairpin ribozyme genes is related to an increase in blood acetaldehyde and its dysphoric effects.

Effects on hepatic activity of ALDH2 and other ALDHs. Enzymatic activity of ALDH2 and of all ALDHs was measured in liver homogenates of treated rats to determine if the increase in acetaldehyde is indeed related to a reduction in ALDH2 activity and, if so, whether it is specific. Upon acetaldehyde determination, i.e., 39 or 41 days after the tail injections, livers were removed and the animals were sacrificed. Rats that received Ad-control had a specific activity of ALDH2 of 14.1 nmoles/mg/minute (**Figure 7**) while animals receiving Ad-Rz or Ad-cRz had specific activities of 10.2 and 10.9 nmoles/mg/min

respectively, representing reductions of 25.4% ($P < 0.01$) and 22.4% ($P < 0.02$) in ALDH2 activity. The minor difference between groups receiving the ribozyme and the control ribozyme genes was not significant ($P > 0.05$) nor was the difference between the two control groups (control adenovirus and vehicle). Total ALDH activity values were between 33.1 and 35.8 nmoles/mg/minute; there were no significant differences between any two experimental groups. These results show that the reduction in alcohol intake in rats that received either ribozyme gene was due to a reduction in ALDH2 activity and the ensuing acetaldehyde increase upon alcohol consumption.

Discussion

Hammerhead ribozymes

In spite of leading to the design of a hairpin ribozyme which was very effective *in vivo*, the fact that the hammerhead ribozyme constructs did not perform as expected warrants reviewing their design.

The effectiveness of hammerhead ribozymes having arms of 8 nt has been shown experimentally,^{24,25} reason for which ribozymes RzUUC9 and RzGUC19 were designed with arms of this length; the 3' arm of RzUUC9 acquired an extra nucleotide from the *HindIII* cloning site. The T_m for the binding of these ribozymes to their targets is ~50°C. RzGUC20 was designed with arms of 10 nt to counteract the complementarity of 6 nt between the arms, giving a T_m of ~60°C, which may account for its greater blocking effect when compared with the other two ribozymes.

Cleavage of the mRNA substrate *in vitro* by RzUUC9 and RzGUC20 was partial, perhaps due to alternative conformations of the substrate. Although the ribozyme:substrate ratio used may seem excessive, there are other studies reporting the use of 5:1, and even 150:1, for ribozymes which were later shown to be active in mammalian cells in culture.^{26–28} The Mg^{2+} , K^+ , and Na^+ concentrations used *in vitro* (10, 140, and 10 mmol/l) closely resemble those described for mammalian cells (0.5, 140, and 10 mmol/l),²⁹ optimal Mg^{2+} differing the most. RzGUC19 did not cut the substrate under these conditions; unavailability of its site *in vitro* is a possibility, since the oligonucleotide used in the accessibility assay binds to nine of the 17 nt of the ribozyme target sequence.

The gene construct encoding a ribozyme may be as decisive for its biological activity as the properties of the ribozyme *per se*. The choice of an RNA pol II promoter allows colocalization of substrate and ribozyme, an important factor for ribozyme action. The target for RzUUC9 is encoded in exon 5, that for RzGUC19 in exon 12 and that for RzGUC20 in exons 12 and 13. The pAC-CMV plasmid has been used in other studies to express ribozyme genes in animal cells in culture²⁵ and *in vivo*.²⁴ The sequence of the expression cassette (GenBank AY590429) predicts that the ribozyme sequences should be preceded by 21 or 22 nt and followed by at least 345 nt. Thus, the 38–42 nt hammerhead ribozymes are embedded in transcripts of > 404 nt. Hence, unforeseen interactions between substrate and ribozyme, and/or negative effects of vector sequence RNA on the hammerhead ribozymes,²⁷ may result in inefficient silencing in mammalian

cells. It is well known that the cellular environment may influence the folding³⁰ and the activity of a ribozyme.^{31–34} The influence of the molecular context is also documented: there is evidence that *cis* cleavage of ribozyme containing transcripts has strong impact on ribozyme *trans* targeting efficiency.³⁵ The limited effectiveness of hammerhead ribozymes in cells may also be due to the lack of a key tertiary interaction present in the extended or natural ribozyme structure.³⁶

Hammerhead versus hairpin ribozymes targeted to the GUC20 triplet of the ALDH2 mRNA

The hairpin RzCG20 and the hammerhead RzGUC20 ribozymes are targeted to the same triplet and are embedded in the same molecular context when cloned in the same vector. Thus, their effects are directly comparable in the pAC-CMV constructs. The results of diminished ALDH2 activity in rat hepatoma cells show that the hairpin ribozyme is twice as effective as the hammerhead ribozyme, corroborating some comparative reports.^{37,38} These differences may be rooted in attributes of the hairpin ribozyme such as its kinetic parameters,³⁵ greater capacity to dissociate from the substrate, and independence of magnesium concentration for the catalysis itself. In this study, the RzGUC20 hammerhead ribozyme only displayed blockade activity while the RzCG20 hairpin ribozyme has a similar blockade capacity but has, additionally, a cleavage ability which may explain its superiority in silencing ALDH2 gene expression.

The hairpin and hammerhead ribozymes used in this study probably differ in the ease with which they dissociate from the substrate. After cleavage, the hairpin ribozyme may remain bound to the mRNA through a 5bp arm and an 11bp arm, three of which are noncanonical pairs (Figure 4). The hammerhead ribozyme may remain bound by 10 canonical base pairs on either side of the substrate (Figure 2). Thus, the hairpin ribozyme may be released more easily, minimizing religation which only requires that both substrate halves remain bound to the ribozyme long enough.^{39–41} Indeed, it has been reported that the reversible cleavage reaction performed by the hammerhead ribozyme is actually favored in the religation direction³⁹ and that the hairpin ribozyme is especially efficient in releasing its substrate.⁴²

In general, ribozymes require magnesium for correct folding and for catalysis *per se*^{23,43} but, although magnesium is essential for the hammerhead ribozyme to catalyze cleavage, it is not required for catalysis by the hairpin ribozyme.⁴⁴ Thus, the intracellular concentration of free magnesium ion in rat hepatoma cells in culture may be too low for the RzGUC20 hammerhead ribozyme, which showed best *in vitro* cleavage at 10 mmol/l; the value reported for rat liver is ~1 mmol/l.⁴⁵

Hairpin ribozyme: molecular context effects

Direct comparison of percent reductions in ALDH2 activity indicates that the RzCG20 hairpin ribozyme is only 15% more effective in the context of the pAC-CMV construct than in the pAAV-CMV vector. Nevertheless, all transfections were carried out with 2 µg of plasmid and, since pAC-CMV is about double the size of pAAV-CMV (9.1 kb versus 4.8 kb), RzCG20 is approximately twice as efficient when in the molecular context provided by pAC-CMV. Given that there are no differences between the two CMV promoters, equal transcription

levels may be assumed. Thus, the differences in the hairpin ribozyme silencing activity between molecular contexts probably lie in the RNA elements upstream and/or downstream of the ribozyme.

There are 22 nt preceding the ribozyme in the pAC-CMV transcript and 590 nt in the unspliced pAAV-CMV transcript (98 nt if the β-globin intron is removed). The regions downstream from the ribozyme also differ in length, ~341 versus ~121 nt respectively, and have different origins: the polyadenylation regions belong to SV40 and to the human growth hormone gene, respectively, such that the efficiency of polyadenylation (and its consequences) may differ as well.⁴⁶ Thus, the flanking regions are likely to have positive or negative effects, possibly due to structure, recruitment of cellular factors and/or the efficiency of polyadenylation and transport to the cytoplasm. However, the ribozyme retains both modes of action, cleavage and blockade, but in different ratios: the pAAV-CMV transcript is associated to greater blockade and less cleavage, perhaps by contributing to binding of the ribozyme to the ALDH2 mRNA and diminishing dissociation of the ribozyme upon cleavage.

Effect of the RzCG20 ribozyme versus that of the cRzCG20 control ribozyme *in vivo*

The only difference observed between the ribozyme and control ribozyme groups in alcohol intake was during the first 15 days but upon stabilization of daily drinking both groups were equal. Although the ribozyme was expected to have a greater effect than the control ribozyme *in vivo* there were no overall differences in alcohol intake, blood acetaldehyde levels, or hepatic ALDH2 activity. Possible explanations are: (i) *in vivo* mechanism for ribozyme action, (ii) the contribution of other ALDHs to blood acetaldehyde, and (iii) differences in the number of infective particles between the adenoviral vectors.

The ribozyme may be unable to cleave the substrate *in vivo* or its cleavage capacity may be fully neutralized by an overly efficient religation reaction; extremely unlikely is the possibility that the control ribozyme may actually cleave the substrate *in vivo* because G8 is part of the active site and required for the acid-base catalysis.⁴⁷ Although mechanistic differences between the ribozymes may actually exist, they may not be apparent in terms of ALDH2 activity 38 days after injecting the viral vectors.

The experimental data presented herein provide direct and indirect evidence of the functionality of the hairpin ribozyme. Thus, given that (i) the hairpin ribozyme and its control ribozyme behave as expected *in vitro* (Figure 4), *i.e.*, RzCG20 cleaves the ALDH2 mRNA, while cRzCG20 does not, and that (ii) cleavage does occur in rat hepatoma cells in culture, as may be inferred from the results shown for the transfection experiments (Figure 3), it is extremely likely that cleavage occurs *in vivo* also. However, this may or may not be the case, so inhibition of mRNA expression by an antisense mechanism alone cannot be dismissed. To answer this question the mRNA levels would have to be measured, either by Northern blot analysis or reverse transcription followed by a polymerase chain reaction (RT-PCR). Northern analysis is not advised because the size of the rat *Aldh2* transcript is very close to that of the 18S rRNA. RT-PCR analysis has not been done. Being this a mechanistic issue, it is certainly

of interest but, regardless of whether RzCG20 does or does not cleave its substrate in the rat liver, the outcome is equally convincing in behavioral and biochemical terms. This study shows that hairpin ribozyme genes are an excellent way to achieve mRNA silencing via antisense recognition: it affords an advantage over an antisense gene (the much smaller size of the target region involved in antisense recognition provides greater specificity) and an advantage over antisense oligonucleotides (gene delivery provides extended duration of the effect).

The relationship between increased blood acetaldehyde and reduced alcohol intake has been documented,^{48,49} and was observed here for both ribozymes. Accordingly, a greater increase in blood acetaldehyde is expected to lead to a greater reduction in alcohol intake. This was the case for one animal which received the RzCG20 gene: it showed the greatest reduction in drinking (60%) and the highest acetaldehyde level 5 minutes after the i.p. alcohol injection (30 % greater than the average). However, this clear relationship between drinking and acetaldehyde does not hold equally for drinking and ALDH2 activity.

In a previous study, in which an ALDH2 antisense gene was delivered to UChB rats in an adenoviral vector,¹⁷ 50% reduction in drinking and 85% reduction in ALDH2 activity were attained 34 days after viral transduction. In this work, drinking was also curtailed 50% but with 24% reduction in enzymatic activity only, showing it is unnecessary to aim for a greater reduction in ALDH2 activity to reach acetaldehyde levels capable of eliciting significant reductions in alcohol intake. The activity of other ALDHs may also have an effect on blood acetaldehyde levels, in particular, those having K_m s for acetaldehyde below 60 $\mu\text{mol/l}$, for example, ALDH1 which has a K_m of 17 $\mu\text{mol/l}$.⁵⁰ Although some rat strains do not have appreciable levels of ALDH1 because of a mutation in the promoter,⁵¹ the genetic diversity of the Wistar strain allows the possibility that there may be ALDH1 activity in UChB rats. Ribozymes diminish ALDH2 activity specifically: there was no reduction in total ALDH activity, to which ALDH2 contributes ~40%, such that a 24% drop in ALDH2 activity represents 9.6% in total ALDH activity and may not have been detected as a significant reduction.

Roughly equal behavioral and biochemical *in vivo* effects for ribozyme and control ribozyme genes may also be due to differences in the viral titers: the viral dose was applied in viral particles, such that more infective particles in the Ad-cRz preparation could compensate the mechanistic superiority of the nonmutant ribozyme seen in cells in culture. Conversely, more infective particles in the Ad-Rz preparation could account for the greater effect in drinking seen for the ribozyme during the first 15 days, even if there were to be no mechanistic differences between the ribozyme and its control ribozyme *in vivo*. To ascertain if there were differences in the infectivity of the two preparations the levels of each ribozyme RNA would also have to be measured in the liver samples of treated animals.

All animals presented a cyclic pattern of alcohol intake during the first two weeks. This behavior may be explained by the need to associate alcohol intake to the aversive effect or by the fact that the adenovirus requires 5–7 days to shed its

shell entirely and reach the maximum expression of a therapeutic gene.⁵² Additionally, the activity of preformed enzyme may not be suppressed by a ribozyme and time is required for its decay; although the half-life of ALDH2 is known for rat hepatoma cells, it has not been determined for the rat *in vivo*.

Silencing ALDH2: utility in curtailing alcohol drinking versus risk of cancer

Many researchers have studied the effect of alcohol on the risk of cancer.⁵³ Alcohol contained in alcoholic beverages has been classified as carcinogenic in humans, and a causal relationship has been established between alcohol intake and cancer of various tissues (breast, mouth, pharynx, esophagus, liver, colon, and rectum) suggesting that the metabolism of alcohol is an important factor in carcinogenesis. Acetaldehyde, the main metabolite of alcohol, and other reactive species generated by the activity of cytochrome 2E1, may lead to DNA damage that, if not repaired, may lead to carcinogenesis. A growing number of studies suggest that both the genetic susceptibility to alcoholism (genetic variations in enzymes of alcohol metabolism) and alcohol intake play a role in the risk of cancer.

Asians who drink alcohol in spite of having a mutant ALDH2 (genotype $ALDH2^{*2}/^{*2}$ or $ALDH2^{*1}/^{*2}$) have an increased risk of developing cancer of the esophagus compared with individuals of $ALDH2^{*1}/^{*1}$ genotype.⁵⁴ Likewise, heterozygous drinkers ($ALDH2^{*1}/^{*2}$) have a greater risk of having mouth or pharynx cancer.⁵⁵ Thus, the inability to degrade acetaldehyde has been associated to a greater cancer risk, demanding caution when considering the inhibition of ALDH2 gene expression as a therapeutic option for alcoholism.

Nevertheless, the purpose of this approach is to inhibit ALDH2 synthesis in the liver only, without affecting the availability of the enzyme in other tissues. Furthermore, ribozyme genes only attain a 24% reduction in ALDH2 activity in rats, minimizing the risks but allowing the desired effects on alcohol intake. It follows that a gene therapy with a ribozyme gene should be safer than with a gene encoding a long antisense RNA which results in 80% reduction in ALDH2 activity, possibly silencing other RNAs. If compared with short antisense RNA, ribozymes appear more advantageous in terms of stability, given their secondary and tertiary structure, and specificity, given that they have two helical elements (instead of one) as the basis for substrate recognition, being less likely to silence RNAs to which they bind imperfectly.

Adenoviral vectors are useful tools for laboratory studies but nonviral vectors may be considered to deliver ribozymes specifically to the liver. For example, Dynamic PolyConjugates⁵⁶ have been shown to deliver RNA molecules to hepatocytes in the rat without evidence of toxicity. Having a specific vector for the liver suppresses the innate immune response against viral vectors by circumventing other organs such as the spleen.⁵⁷

Overall, these experiments in alcohol dependent UChB rats transduced with adenoviral vectors show that a hairpin ribozyme recognizing the ALDH2 mRNA by only 16bp may silence its substrate *in vivo*, leading to a reduction in voluntary alcohol intake similar to that obtained with an adenoviral vector encoding an antisense RNA complementary to the whole mRNA (>1800 nt),¹⁷ representing a substantial improvement in specificity.

Materials and methods

Gene constructs. The Sprague–Dawley rat cDNA for ALDH2 (nts 32–1874; GenBank X14977) was amplified by PCR from plasmid p19NSALDH (a gift from Henry Weiner) and cloned in pGEM-T Easy (Promega, Madison, WI) by direct cloning to generate plasmid pCMBZ. Hammerhead ribozyme gene constructs were generated by cloning a mixed duplex having cohesive ends encoding each ribozyme and its control ribozyme in pGEM-3Zf(+) (Promega) for *in vitro* experiments and in pACCMVpLpARS(+)⁵⁸ for cell culture experiments. Each duplex was obtained by annealing two mixed sequence oligonucleotides encoding each wild type and control ribozyme pair. Duplex sequences are: UUC9/**GAA TTC TAG CTG AAC TGA TGM GTC CGT GAG GAC GAA AAG TCG CCA AGC TT**; GUC19/**GAA TTC GCT CCC CCT GAT GMG TCC GTG AGG ACG AAA CAT CTT AAG CTT**; GUC20/**GAA TTC TTT GAC GGT CTG ATG MGT CCG TGA GGA CGA AAC CGT CTT CAA GCTT** (full *EcoRI-HindIII* recognition sites shown, ribozyme encoding sequences in bold). Hairpin ribozyme gene constructs were made by annealing two overlapping oligonucleotides and filling in with Klenow to obtain a blunt mixed sequence duplex (**CCT GAA TTC TTG ACG GTA SAA GTC TAC CAG AGA AAC ACT CTT CGG AGG TAT ATT ACC TGG TAA GCT TGT CTG AG**) which was subjected to *EcoRI* and *HindIII* digestion and cloned in pGEM-3Zf(+) for *in vitro* experiments, and in pACCMVpLpARS(+) or pAAV-MCS (Stratagene) for cell culture experiments; these parent plasmids are referred to as pAC-CMV and pAAV-CMV respectively. The *NotI-NotI* gene cassettes from the pAC-CMV plasmid and the RzCG20 and its control ribozyme derivatives were transferred to the *NotI* site of pShuttle, a precursor plasmid for the preparation of adenoviral vectors (see below). Clones carrying the genes of interest in the same direction as the kanamycin resistance gene were chosen for adenoviral production. All gene constructs generated in this work were confirmed by sequencing.

RNA synthesis. Rat ALDH2 mRNA and hammerhead ribozymes were obtained by *in vitro* run-off transcription with T7 RNA polymerase (Promega); templates were linearized with *SaI* or *HindIII* respectively. Typically, 50 μ l standard reactions using 2.5 μ g of template were carried out for 1 hour at 37°C. DNA templates were eliminated by extraction with phenol saturated in water; RNA was precipitated with ethanol and sodium acetate and resuspended in 50 μ l of water.

Accessibility assays. ALDH2 mRNA (5 μ l) was renatured in 7 μ l of 40 mmol/l Tris-HCl pH 7.5, 4 mmol/l MgCl₂ and 1 mmol/l dithiothreitol (DTT) by incubation at 70°C for 5 minutes and slow cooling to 37°C. Annealing of each DNA oligonucleotide (75 pmoles) was attained by incubation at 37°C for 10 minutes. RNase H was added (0.5 U, GibcoBRL, Bethesda, MD); digestion proceeded in 10 μ l at 37°C for 15 minutes. After extraction and precipitation, reaction products were resuspended in 9 μ l of water. Reverse transcription was performed in 25 μ l at 42°C for 60 minutes by addition of 65 pmoles of unlabeled primer, 10 pmoles of 5' ³²P labeled primer (~8 × 10⁵ cpm), 200 u of M-MLV reverse transcriptase, 2 mmol/l each dNTP in 50 mmol/l Tris-HCl pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl₂, and

10 mmol/l DTT in a final volume of 25 μ l; primer and RNA were first denatured together at 70°C for 10 minutes. cDNA was precipitated, resuspended in 10 μ l, and analyzed by autoradiography after electrophoresis in denaturing 5% polyacrylamide gels which were transferred to Whatman 3 MM paper.

Primers (150 pmoles) were labeled on the 5' end using 10 u of T4 kinase and 10 μ Ci of [³²P]-ATP (3.3 pmoles/ μ l) in 70 mmol/l Tris-HCl pH 7.6, 10 mmol/l MgCl₂, 100 mmol/l KCl and 1 mmol/l 2-mercaptoethanol for 40 minutes at 37°C. After heat inactivation of T4 kinase (65°C, 10 minutes), unincorporated radionuclide was removed by precipitation (ethanol/sodium acetate, 20 minutes incubation at -20°C, 15 minutes centrifugation at 20,817 \times g at 4°C). Primers were washed with 75% ethanol, dried, and resuspended in 25 μ l of water; recovery was estimated at 85%.

***In vitro* cleavage activity of hammerhead and hairpin ribozymes.** The ALDH2 mRNA (6.4 pmoles) was incubated with each hammerhead ribozyme (32 pmoles) in 10 μ l of 50 mmol/l Tris-HCl pH 7.4, 10 mmol/l MgCl₂, 10 mmol/l NaCl, and 140 mmol/l KCl overnight at 37°C; RNA substrate and ribozymes were renatured separately by a previous 15 minutes incubation at 50°C followed by slow cooling to 37°C. Cleavage products were visualized in denaturing polyacrylamide gels stained with ethidium bromide. Hairpin ribozymes were tested analogously.

Lipofection of H4-II-E-C3 rat hepatoma cells. Cells (ATCC CRL-1,600) were grown in 2 ml of Dulbecco's modified Eagle medium (DMEM 12800-0187, Gibco, Invitrogen Corporation, Grand Island, NY), supplemented with 1.5 g/l sodium bicarbonate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5% characterized bovine fetal serum (Hyclone) and 10% defined equine serum (Hyclone, Logan, UT) at 37°C and 5% CO₂; 1 × 10⁶ cells were plated in 35 mm wells or plates. Upon reaching 60–70% confluence (18–20 hours), cells were washed 3 times with medium lacking sera and antibiotics (transfection medium). Lipofections were carried out in 2 ml of this medium using cationic liposomes as transfection reagent. Plasmids were purified with Wizard Plasmid Midipreps (Promega) and quantified by absorbance at 260 nm. DNA (2 μ g per transfection) was diluted in 120 μ l of transfection medium to which 5 μ l of Lipofectamine 2000 (Invitrogen) were then added. The mixture was maintained at room temperature for 20 minutes prior to its addition to the cells. Following 5 hours of incubation, the transfection medium was removed and replaced with whole medium. Cells were grown for an additional 43 hours, reaching 100% confluence. The medium was removed, and plates were frozen at -80°C and thawed; 500 μ l of 1% Triton X-100 and 1 mmol/l ethylenediaminetetraacetate were added, and plates were shaken at 500 \times g at 4°C for 15 minutes. Cell lysates were cleared by centrifugation (4°C, 15 minutes, 1,000 \times g) and supernatants were used to determine enzymatic activity (150 μ l aliquots); protein concentrations were determined with the MicroBCA Assay (Pierce, Rockford, IL) in 5 μ l aliquots reading absorbance at 562 nm.

Preparation of adenoviral vectors. First generation adenoviral vectors (Δ E1 and Δ E3) were generated using the AdEasy Basic Kit (Stratagene, La Jolla, CA).⁵⁹ Recombination between

shuttle plasmids (see above; pShuttle carrying the gene cassettes encoding RzGC20, cRzCG20, and the nonfunctional RNA, 7.9kb, linearized with *PmeI*) and pAdEasy-1 (~33.4 kb) was carried out in *E. coli* BJ5183-AD-1 (a recombinogenic strain) under Km selection; clones were screened by *PacI*, *EcoRI*, and *BamHI* digestion. Shuttle plasmids (~150 ng) were incorporated into bacteria (containing pAdEasy-1) by electroporation. Candidate plasmids (~35.5 kb) were screened by digestion with *EcoRI*, *BclI*, and *SnaBI*. Recombinant plasmids were amplified in *E. coli* DH5 by transformation using the CaCl_2 method and purified from 200 ml cultures using the Jet-Star 2.0 system midi columns (Genomed, Löhne, Germany).

Adenoviral vectors were generated in the human embryonic kidney cell line HEK293 (ATCC CRL1573) cultured in DMEM as indicated above, but using only fetal bovine serum (10%). Cells (2×10^6) were plated in 25 cm² flasks in 5 ml of whole medium. Upon reaching 50% confluence (~24 hours) cells were transfected, in the absence of serum or antibiotics, with each recombinant adenoviral plasmid (4 μg) linearized with *PacI* and prepared appropriately in Lipofectamine 2000 (Invitrogen) (20 μl) in a 3 ml volume for 5 hours.⁶⁰ Medium complements were restored, the medium was changed 48 hours later, and cultures were prolonged up to 15 days total. Cells were harvested, resuspended in 2 ml of supernatant and lysed with four cycles of freeze/thawing (ethanol bath at $-80^\circ\text{C}/37^\circ\text{C}$ water bath); lysates were cleared by centrifugation and stored at -80°C . Adenoviral vectors were amplified sequentially in four steps of increasing culture size (1 \times 25 cm² flask; 1, 3, and 40 \times 75 cm² flasks). Viral particles were purified by two consecutive CsCl gradients (discontinuous and continuous) and the resulting 1.5 ml volume was dialyzed in a Slide-A-Lyzer cassette (Thermo Scientific, Rockford, IL) of 10 kDa pore size and 3 ml capacity against 10 mmol/l Tris-HCl pH 8, 2 mmol/l MgCl₂ and 5% sucrose at 4°C.

Identity of each adenoviral vector preparation was confirmed by restriction enzyme analysis (*MbolI*, *BamHI*, and *RsaI*) of an 860 bp amplicon (RzCG20 and cRzCG20) or 853 bp amplicon (control RNA) contained in the *NotI* gene cassette (1277 or 1270 bp) obtained by PCR of 170 fg of recombinant adenoviral DNA using primers TG-34 (GTT CCC ATA GTA ACG CCA ATA) and TG-233 (ATT TGT AAC CAT TAT AAG CTG C) anchored to the CMV promoter and SV40 polyadenylation sequence respectively. Viral titers (vp/ml) were estimated as described.⁶¹

In vivo experiments. UChB rats (*Universidad de Chile-Bibulous*) have been selected continuously since 1948 from the Wistar strain by their high voluntary alcohol consumption.¹⁶ Female 2-month old rats, ~200 g in weight of generations 80 or 81 and *Aldh2¹/Aldh2²* genotype (GenBank AY566467) kept in a 12 hours light/dark cycle were used. Procedures were approved by the Faculty of Medicine and Faculty of Chemical and Pharmaceutical Sciences ethics institutional boards and by the Chilean National Science and Technology Research Commission (CONICYT).

Rats were allowed free choice between a 10% v/v ethanol solution and water which were permanently available in two graduated tubes for 30 days; food was also available *ad libitum*. Once stabilized, ethanol intake was calculated from the average of the last 14 days. Access to ethanol was suspended

and viral preparations or dialysis buffer were injected in the tail vein using Ultra-Fine 29G 13 mm needles (Becton Dickinson). Free choice (ethanol availability) was resumed 96 hours later during one hour a day (limited access) during the light cycle.

Blood acetaldehyde measurements. Detoxified rats were anesthetized by intra muscular injection of 2 mg/kg acepromazine (Drug-Pharma, Invetec, Santiago, Chile) and, 10 minutes later, 60 mg/kg ketamine (Laboratorio Richmond, Buenos Aires, Argentina) in the back leg. Alcohol was then administered i.p. (1 g/kg, in a 20% v/v solution) and arterial acetaldehyde was determined at 0, 5, 10, 15, and 30 minutes in 0.1 ml blood samples obtained from the carotid artery exposed by surgery. Sampled blood was added to 0.9 ml of distilled water at 4°C kept in 50 ml serum glass vials (Wheaton, Millville, NJ) hermetically sealed with Mininert valves (Supelco, Bellefonte, PA) kept in ice. Vials were incubated 15 minutes in ice and 15 minutes in a water bath at 60°C, and 1 ml of gas phase was drawn to measure acetaldehyde. Analysis was done by head space gas chromatography as described⁴⁹ using an SRI 8610 instrument (SRI Instruments, Las Vegas, NV), a 5% Carbowax 20M over Carbowax 60/80 column (Alltech Associates, Chicago, IL), a nitrogen flow of 65 ml/min, and a flame ionization detector.

ALDH2 activity measurements. Supernatants of H4-II-E-C3 cell lysates were mixed with 14 $\mu\text{mol/l}$ propionaldehyde and 800 $\mu\text{mol/l}$ NAD⁺ in 34 $\mu\text{mol/l}$ dibasic sodium phosphate pH 8.5, 4 mmol/l DTT, 5 mmol/l MgCl₂, 1 mmol/l 4-methyl pyrazole (an alcohol dehydrogenase inhibitor that prevents propionaldehyde being reduced to alcohol) or 10 mmol/l pyrazole, and 10 $\mu\text{mol/l}$ NADH in 800 μl . Reactions were performed at 35°C, registering absorbance readings at 340 nm (NADH). Samples were incubated 10 minutes with NAD⁺ and NADH in the absence of substrate to deplete other activities that generate or use NADH, propionaldehyde was then added and the reaction was followed another 15 minutes. Initial rates and specific ALDH2 activities were calculated; values ranged between 0.5 and 2 nmoles NADH/mg protein/min.

Liver samples from treated rats were prepared as follows: 500 mg of tissue were washed 3 times with 2.5 ml of cold phosphate-buffered saline, transferred to a 15 ml glass Corex tube and homogenized in 3 ml of cold phosphate-buffered saline with 5 rounds of 30 seconds with an Ultra Turrax T25 homogenizer (Janke & Kinkel, IKA Labortechnik, Staufen, Germany) and an incubation of 1 minute in ice between rounds. After centrifugation at 12,000 $\times g$ for 20 minutes at 4°C, the supernatant (containing hemoglobin) was discarded, and the pellet, which contains mitochondria, was homogenized as indicated in 2.5 ml of cold 1% Triton X-100 and 0.33 mmol/l DTT in Na₂HPO₄ pH 7.4. The suspension was clarified by centrifugation at 1,000 $\times g$ for 10 minutes at 4°C; 2 ml of supernatant were then centrifuged at 20,800 $\times g$ for 20 minutes at 4°C. Activity measurements were carried out in 500 μl of supernatant; 10 μl were used to determine protein concentration. These aliquots were frozen at -80°C for at least one night. The entire procedure was performed twice on different days.

The ALDH2 activity of liver homogenates was measured in samples of 350 μg of protein content (typically, 24–36.5

μl) which were mixed with 4 mmol/l DTT, 2.5 mmol/l MgCl₂, 800 μmol/l NAD⁺, 10 μmol/l NADH, 10 mmol/l pyrazole and 14 μmol/l propionaldehyde in 46 mmol/l NaH₂PO₄ buffer pH 7.4 in 800 μl. The rest of the procedure was as indicated above. To determine the total ALDH activity of the liver lysates, the reaction was carried out at 1 mmol/l propionaldehyde.

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