

# Inhibition of luciferase expression by synthetic hammerhead ribozymes and their cellular uptake

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## ABSTRACT

**Two synthetic hammerhead ribozymes, one unmodified and the other with 2'-modifications and four phosphorothioate groups, targeting a single GUA site in the luciferase mRNA, were compared for their inhibition of gene expression in cell culture and their cellular uptake was also analysed. A HeLa X1/5 cell line stably expressing luciferase, under an inducible promoter, was treated with these ribozymes by liposome-mediated transfection to determine their activity. Luciferase expression in cells was inhibited to ~50% with little difference between the unmodified and the 2'-modified ribozyme. A similar degree of inhibition was observed with two catalytically inactive ribozymes, indicating that inhibition was mainly due to an anti-sense effect. A ribozyme carrying a cholesterol moiety, applied to the cells without carrier, showed no inhibition. Northern blotting indicated a similar amount of cellular uptake of all ribozymes. The unmodified ribozyme was essentially evenly distributed between cytoplasm and nucleus, whereas a higher proportion of the phosphorothioate-containing ribozyme was observed in the nucleus. Fluorescence microscopy, including confocal microscopy using 5'-fluorescein-labelled ribozymes, showed that the unmodified and 2'-modified ribozymes were present in the cytoplasm and in the nucleus to a similar extent, whereas the fluorescence of the phosphorothioate-containing ribozyme was much stronger in the nucleus. Both ribozymes inhibited luciferase expression to a comparable degree, suggesting that the ribozyme in the nucleus did not contribute significantly to the inhibition. Ribozymes with a cholesterol moiety were predominantly trapped in the cell membrane, explaining their inability to interfere with gene expression.**

## INTRODUCTION

Ribozymes are RNA molecules that can inhibit gene expression by catalysing RNA cleavage in a sequence-specific manner. This property has prompted many studies to develop ribozymes as

potential therapeutic agents in treating diseases, mainly cancers and viral infections (for reviews see 1–3). One of the major problems in the therapeutic application of ribozymes is their efficient cellular delivery. Current strategies include the exogenous and the endogenous delivery. In the former approach, preformed ribozymes are delivered to the cell via microinjection or by a transfection procedure. However, this strategy is limited by the poor nuclease stability of ribozymes. For this reason most ribozyme studies have employed the second strategy, where a plasmid or viral vector is used to introduce the ribozyme gene into the cells and have the ribozyme expressed intracellularly by transcription. However, synthesis of chemically modified ribozymes has resulted in a considerable increase in resistance against nuclease degradation, without compromising catalytic activity (reviewed in 1). This has led to a number of examples for exogenously delivered hammerhead ribozymes for the inhibition of gene expression studies in cell culture using 2'-modified ribozymes (4–8), or ribozymes stabilised by incorporation of deoxynucleotides or phosphorothioates (9–15), but also unmodified ribozymes (16–19). 2'-Modified ribozymes and an unmodified ribozyme have also been used in animal studies (20–22).

A major obstacle limiting the exogenous delivery of ribozymes is their polyanionic character which prevents efficient diffusion through biological membranes. This property is common to oligodeoxynucleotides which are used in the anti-sense technology. Cationic lipids are most commonly used as carriers to introduce such oligonucleotides into cells. Several formulations, containing different cationic lipids, have been successfully used to deliver oligodeoxynucleotides for anti-sense inhibition of gene expression (23–25). Alternatively, conjugation of oligodeoxynucleotides with polylysine (26), geraniol (27) or cholesterol has been shown to improve cellular uptake and consequently, the efficacy of inhibition of gene expression (28–33).

In the present study we have investigated the importance for chemical modification of ribozymes for the inhibition of gene expression in cell culture, their cellular uptake and localisation and the possibility to deliver ribozymes without cationic lipids by conjugating them with cholesterol. Various ribozymes, unmodified or chemically modified, directed at a single position in the luciferase gene, were studied in their *in vitro* activity to cleave the luciferase transcript and their ability to interfere with luciferase expression in cell culture. A HeLa X1/5 cell line which stably expresses the luciferase gene under a tetracycline-regulated

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promoter (34), was transfected by lipofection with these ribozymes. Additionally, ribozymes with a cholesterol modification were delivered to the cells without a carrier to determine their inhibitory effect. Ribozymes were 5'-labelled with fluorescein and their cellular uptake patterns were determined by fluorescence microscopy and confocal laser scanning microscopy (CLSM).

## MATERIALS AND METHODS

Phosphoramidites were purchased from PerSeptive Biosystems. Fluorescein-phosphoramidite was obtained from Pharmacia. T7 Polymerase was purified from the overproducer *Escherichia coli* BL 21/pAR1219 kindly supplied by F. W. Studier (Brookhaven). [ $\alpha$ - $^{32}$ P]UTP, [ $\gamma$ - $^{32}$ P]ATP, Hybond-N<sup>+</sup> Nucleic acid transfer membrane and ECL-Detection Reagent were purchased from Amersham. Ribonucleoside triphosphates, DNase I (RNase free), restriction endonuclease *Xho*I and its 10 $\times$  buffer were obtained from Boehringer Mannheim. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FCS), bicinchoninic acid, coenzyme A (CoA) and luciferase-protein were purchased from Sigma. Penicillin/streptomycin were obtained from ICN Biomedicine. The HeLa X1/5 cell line was a gift of H. Bujard (Heidelberg, Germany). Tfx-50, beetle luciferin, horseradish peroxidase-conjugated goat anti-rabbit antibody and rabbit anti-luciferase antibody were purchased from Promega. Nitrocellulose transfer membrane (PROTRAN BA 86, 0.34  $\mu$ m) was obtained from Schleicher & Schuell, Ultraspec RNA from Biotecx Laboratories (Houston, TX), pTRI- $\beta$ -actin-human from Ambion and thiocholesterol was from Aldrich.

### Synthesis

Ribozymes were chemically synthesized and purified as described previously (35). MALDI-TOF mass spectral analysis was performed by G. Kuhnle, Leipzig: Rz865 [M+H]<sup>+</sup> calc. 10429.4; found 10436.5; Rz865inact. [M+H]<sup>+</sup> calc. 10413.4; found 10419.6; Rz865M-PS [M+H]<sup>+</sup> calc. 10811.4; found 10879.6; Rz865M-PS inact. [M+H]<sup>+</sup> calc. 10795.5; found 10839.9. The fluorescein-phosphoramidite from Pharmacia was used for the incorporation of the dye as the last step in the oligonucleotide synthesis. The ribozyme with cholesterol-attachment (Rz865Ch) was synthesised using the strategy developed for site-specific labelling of oligoribonucleotides through 2'-aminocytidine by reacting it with an isocyanate (36).

The ribozyme containing a 2'-aminocytidine nucleotide (Rz865-NH<sub>2</sub>) (Fig. 1) was prepared according to Heidenreich *et al.* (35). Ribozyme Rz865-NH<sub>2</sub> (10 A<sub>260</sub> units, 47.34 nmol) was dissolved in 50  $\mu$ l boric acid/borax buffer (0.07 M, pH 8.6), 33.4  $\mu$ l dimethylformamide (DMF) was added and the solution was kept in an ice bath for 10 min. Four equal aliquots of a total of 16.6  $\mu$ l of a 150 mM solution of 2-isocyanatoethyl 2-pyridyl disulfide (37) in DMF (total 2.5  $\mu$ mol) were added over a period of 20 min with stirring and cooling in an ice-salt bath. After 3 h, the reaction mixture was evaporated to dryness and the residue dissolved in 400  $\mu$ l water. This solution was extracted with EtOAc (4 $\times$  500  $\mu$ l) and the aqueous phase evaporated to dryness. The residue was dissolved in water. Reverse-phase HPLC analysis showed the compound to be

90% pure and was used without further purification for the next reaction. [M+H]<sup>+</sup> calc. 10640.7; found 10634.1.

This substituted ribozyme (2 A<sub>260</sub> units, 9.46 nmol) was dissolved in 50  $\mu$ l Tris-HCl buffer (500 mM, pH 8.5), followed by addition of 12  $\mu$ l DMF. To this, 10  $\mu$ l of a preheated solution (56°C, 5 min) of thiocholesterol in DMF (10 mM) was added. The reaction mixture was incubated at 37°C for 3 h with occasional vortexing when HPLC analysis indicated ~65% conversion to product Rz865Ch (retention time for product 19.42 min). The reaction mixture was subjected to preparative reverse-phase HPLC and the product-containing fractions were combined, evaporated to dryness and the residue dissolved in water. Yield was 0.6 A<sub>260</sub> units (30%). [M+H]<sup>+</sup> calc. 10932.3; found 10923.5.

### *In vitro* experiments

For *in vitro* transcription, a plasmid containing the coding sequence of luciferase behind a T7 promoter [pHA-Luc] (38) was linearised with *Xho*I, phenol-extracted and ethanol-precipitated. Linearised plasmid-DNA (3  $\mu$ g), 20 U of RNase inhibitor, 2500 U T7 RNA polymerase and 150  $\mu$ Ci [ $\alpha$ - $^{32}$ P]UTP, were incubated in a volume of 50  $\mu$ l for 1 h at 37°C in a buffer containing 10 mM dithiothreitol (DTT), 500  $\mu$ M of each NTP, 50 mM Tris-HCl, pH 7.5, 2 mM spermidine and 6 mM MgCl<sub>2</sub>. Thereafter, 50 U of DNase I (RNase-free) were added for 15 min. Unincorporated NTPs were removed by centrifugation with microconcentrators Micron-50 (Amicon) according to the protocol provided by the manufacturer. The concentration of the aqueous RNA solution was determined by UV absorption at 260 nm to range from 0.1 to 2.0  $\mu$ M. RNA was stored at -20°C.

Cleavage kinetics with the *in vitro* transcript were performed, under single turnover conditions with a range of ribozyme concentrations. The ribozymes were incubated at 75°C for 1 min in the presence of 50 mM Tris-HCl, pH 7.5. After cooling to 37°C, MgCl<sub>2</sub> was added to a final concentration of 10 mM. Cleavage reactions with 100 nM substrate were performed in a total volume of 25  $\mu$ l, containing 50 mM Tris-HCl, pH 7.5 and 10 mM MgCl<sub>2</sub>. Reaction was initiated by addition of the ribozyme (concentrations between 200 and 1000 nM) and incubated at 37°C for 1 h. Reactions were stopped by the addition of 10  $\mu$ l stop-mix (7 M urea, 50 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). The resulting cleavage products were analysed on a 6% polyacrylamide-8 M urea gel (40 cm, 1 h at 50 W) and the remaining fraction of the substrate was determined by a Fuji Bas-2000 PhosphorImager. Kinetic constants for the cleavage were obtained by plotting the observed cleavage rate,  $k_{\text{obs}}$  against the quotient of  $k_{\text{obs}}$  over the ribozyme concentration  $[E]_g$ , according to the following equation:

$$-\ln(\text{Frac } S)/t = k_{\text{obs}} = -K_M(k_{\text{obs}}/[E]_g) + k_{\text{react}}$$

The reaction rate  $k_{\text{obs}}$  equals the negative logarithm of the remaining substrate (Frac S) divided by the reaction time  $t$ . The negative slope represents the  $K_M$  value, and the intercept of the regression line with the ordinate gives the maximal reaction rate,  $k_{\text{react}}$ , under single turnover conditions (35).

### Cell culture experiments

The HeLa X1/5 cell line stably expresses luciferase and has previously been described in detail (34). Cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml

penicillin and 10 µg/ml streptomycin. Cells were kept in a moist atmosphere with 10% CO<sub>2</sub> at 37°C.

The day before transfection, exponentially growing cells were seeded onto 6-well tissue culture plates according to standard protocols at a density of 2 × 10<sup>5</sup> cells per well, in 3 ml medium containing tetracycline (100 ng/ml). Cells were grown overnight (at least 18 h) to yield ~50–70% confluency for transfection. Prior to transfection, ribozymes and Tfx-50 were complexed in 200 µl DMEM for 20 min (1000 pmol ribozyme/10 µl Tfx-50). Cells were washed with fresh medium with or without FCS and incubated for 2.5 h in 800 µl DMEM, either with or without 10% heat-inactivated FCS, supplemented with the Tfx-50/ribozyme complexes (final concentration of ribozyme 1 µM). Cholesterol-modified ribozymes were diluted in 200 µl DMEM, without Tfx, before adding to the cells (final concentration of ribozyme 3 µM). In this case, transfection was performed in the absence of FCS. After 2.5 h at 37°C cells were washed twice with DMEM (10% FCS). Transfected cells were cultured to 24 h in medium with 5 ng tetracycline to induce expression of luciferase. Results present an average of at least three different experiments, each done in duplicate.

Inhibition of gene expression by ribozymes was determined by measuring the activity of luciferase. At defined time points after transfection, cells were washed twice with phosphate buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and cell extracts were made using 130 µl of lysis buffer.

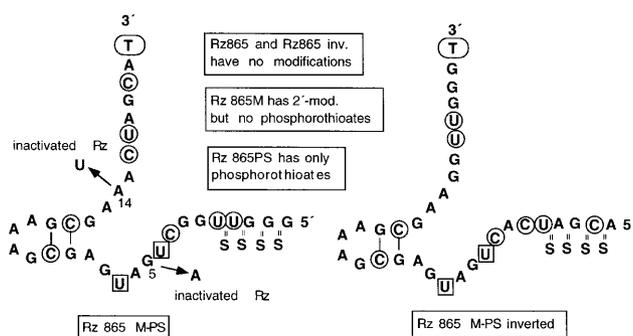
Luciferase expression was quantified with 20 µl of centrifuged lysate supernatant using a buffer containing 20 mM Tricine, 1.07 mM (MgCO<sub>3</sub>)<sub>4</sub>, Mg(OH)<sub>2</sub> × 5H<sub>2</sub>O, 2.67 mM MgSO<sub>4</sub>, 0.1 mM EDTA, 33.3 mM DTT, 270 µM CoA, 470 µM luciferin and 530 µM ATP. Light emission was measured and relative light units (RLU)/mg protein determined by a Lumat LB9501 luminometer (Berthold). Protein concentration was determined using the PIERCE BCA assay according to the protocol supplied by the vendor.

### Western immunoblot

Levels of luciferase protein in cells were determined by western immunoblotting. Cells were plated and transfected as described before. After 18 h growth, cells were washed twice with PBS before lysing them. Protein concentration was determined using BCA reagent (PIERCE) following the manufacturer's protocol. Protein (30 µg) was separated on a 7.5% polyacrylamide gel (8 M urea) and electrotransferred (Trans-Blot Semi-Dry; Bio-Rad) to nitrocellulose transfer membrane. Membrane was probed with rabbit anti-luciferase antibody (1:500) and blots were developed using horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:1000). Protein bands were visualised using ECL detection reagent according to the protocol (Amersham). Membranes were exposed to X-Omat AR film (Eastman Kodak) for 3–60 s.

### Northern blot

Ribozymes in cells were identified by northern blotting. Cells were plated and transfected with ribozymes as described above. After the indicated time of growth, cells were washed twice with PBS. For determination of ribozymes in unfractionated cells, RNA was isolated with Ultraspec RNA™ as described by the manufacturer. For determination separately in the cytoplasm and in the nucleus, cells were resuspended in RNA extraction



**Figure 1.** Sequences and modifications of ribozymes targeted against luciferase mRNA position 865. 2'-Fluoro-2'-deoxypyrimidine nucleotides, circled; 2'-amino-2'-deoxypyrimidine nucleotides, boxed. Phosphorothioate linkages, S; T, inverted deoxythymidine 3'–3' linkage.

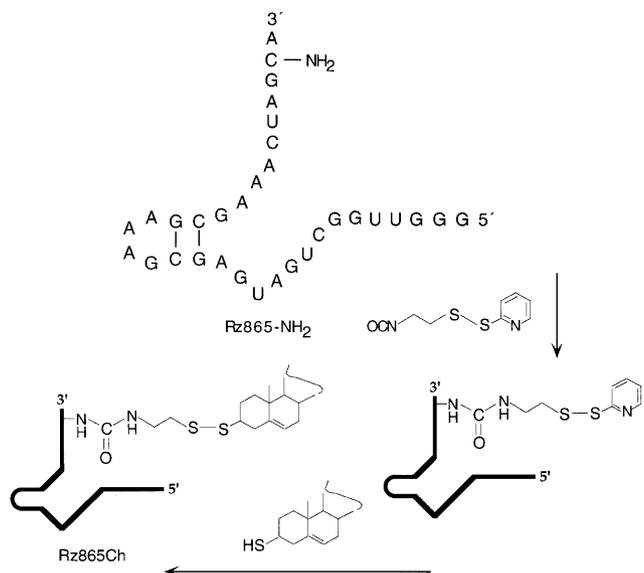
buffer [0.14 M NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.6), 0.5% Nonidet P-40, 1 mM DTT, 1000 U/ml RNase inhibitor]. After 5 min incubation on ice the cytoplasm was separated from the nucleus by a centrifugation step at 12 000 g for 90 min at 4°C. RNA of both fractions was isolated with Ultraspec RNA™ as described by the manufacturer. RNA was separated by a 1.5% (w/v) agarose-formaldehyde gel. Northern transfer and hybridisation was performed according to standard procedures (39). The same blot was probed with a <sup>32</sup>P-end-labelled Rz865-specific oligodeoxynucleotide and an internally-<sup>32</sup>P-labelled β-actin antisense transcript. Ribozyme and β-actin levels were quantitated on a Fuji-Bas-2000 Phosphor-Imager. The β-actin mRNA served as an internal RNA quantitation reference.

### Fluorescence microscopy

Cells (0.5–1 × 10<sup>5</sup>) were grown and transfected with the fluorescein-labelled ribozymes in a 12-well tissue culture plate as described. At defined time points, unfixed cells were observed under a fluorescence microscope (Zeiss Axiovert 100) through a 100× objective and living cells were photographed using a video camera (Kappa CF 20 DXC). For CLSM, cells were grown on coverslips which were removed from culture plates after transfection with fluorescein-labelled ribozymes and placed cell-site down on a drop of PBS:glycerol (1:1) solution. Cells were not fixed to avoid artefacts resulting from the fixation procedure. Intracellular distribution of ribozymes was visualised directly using a CLSM with a krypton/argon laser.

## RESULTS

Four different sites with GUC or GUA triplets, positions 491, 798, 865 and 1177, downstream from the starting AUG codon, were chosen for ribozyme cleavage of the luciferase mRNA transcript. These sites were selected as they were at or near loop regions, as indicated by the Mfold program of the GCG package. Single turnover kinetics with unmodified ribozymes indicated that the ribozyme which cleaved the transcript at position 865 was the most efficient (Table 1). Thus, this ribozyme (Rz865) was selected for further studies (Fig. 1). This ribozyme was also modified by introducing 2'-substituted



**Figure 2.** Synthesis of Rz865Ch. Dark line drawing represents the ribozyme schematically.

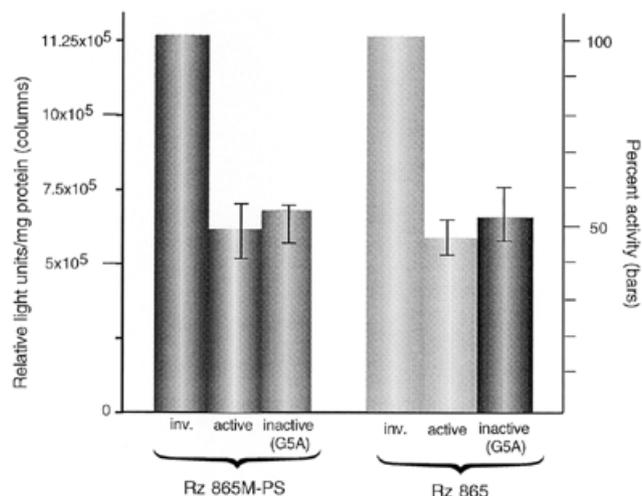
pyrimidine nucleotides and by incorporating four phosphorothioates at the 5' end and an inverted thymidine at the 3' end, to provide stability against nuclease degradation (Rz865M-PS) (4,5,35). The cleavage efficiencies of Rz865 and of its chemically-modified forms compared favourably with published data on related constructs (6,35). In another variant, a cholesterol moiety was attached near the 3' end of Rz865 to study its cellular uptake (Rz865Ch) (Fig. 2). The cleavage activities of these ribozymes were measured using the luciferase transcript as the substrate under single turnover conditions (Table 1). Ribozyme Rz865 showed a somewhat higher degree of cleavage with  $k_{\text{react}}/K_M$   $2428 \text{ M}^{-1} \text{ s}^{-1}$  than the 2'-modified Rz865M-PS with  $975 \text{ M}^{-1} \text{ s}^{-1}$ . The cholesterol ribozyme Rz865Ch with  $1076 \text{ M}^{-1} \text{ s}^{-1}$  was as active as Rz865M-PS.

**Table 1.** Cleavage constants of ribozymes

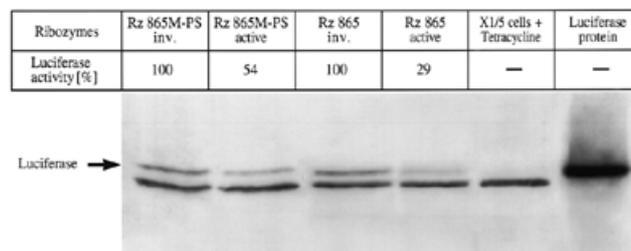
Ribozyme	$k_{\text{react}} [10^{-3} \text{ s}^{-1}]$	$K_M [\text{nM}]$	$k_{\text{react}}/K_M [\text{M}^{-1} \text{ s}^{-1}]$
Rz491	0.22	414	531
Rz798	0.24	278	863
Rz865	0.34	140	2428
Rz865M-PS	0.16	164	975
Rz865M	0.16	160	1000
Rz865Ch	0.14	130	1076
Rz1177	Not detectable		

Ribozymes were incubated with the *in vitro*-transcribed full-length luciferase-RNA for 1 h at 37°C. Cleavage kinetics were performed under single turnover conditions as described in Materials and Methods.

A ribozyme in which the substrate binding arms were inverted (Rz865 inv) served as a control for the sequence specificity of cleavage. Neither this ribozyme nor a catalytically



**Figure 3.** Inhibition of luciferase activity by ribozymes. HeLa X1/5 cells were transfected with 1  $\mu\text{M}$  ribozymes Rz865 and Rz865M-PS and their corresponding controls. After 18 h of growth, the luciferase activity was determined. Luciferase activity of cells treated with the inverted ribozyme was defined as 100%. Measurements were taken in duplicate and results present an average of three different experiments.

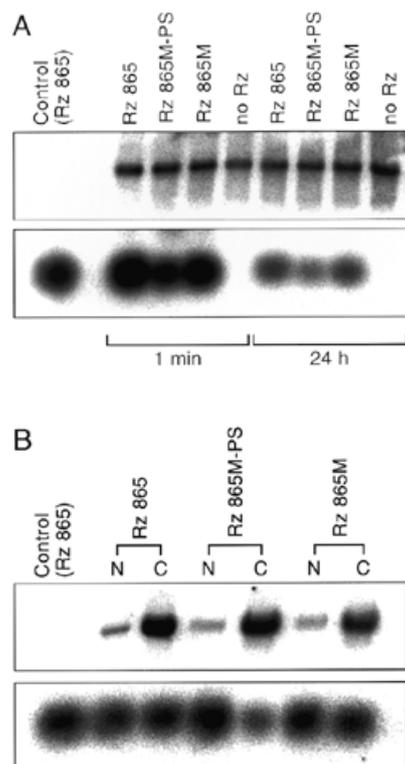


**Figure 4.** Western immunoblot. Amount of luciferase protein in HeLa X1/5 cells treated with active ribozymes and their respective controls. Cells were treated with ribozyme-Tfx-50 complexes for 2.5 h. After PBS washing, cells were grown. After 20 h the cell extract was prepared. The same extract was used to determine luciferase activity and amount of luciferase protein. Extract from cells grown in the presence of tetracycline was used as a control. Purified luciferase protein was used as a standard. The upper band shows luciferase protein; the lower band results from a cross-reaction with cellular proteins. The amount of protein and luciferase activity are compared in the upper section.

incompetent ribozyme (Rz865 inactive), where G at position 5 was exchanged for A, produced any cleavage products.

### Inhibition of luciferase expression in cell culture

X1/5 cells were treated with 1  $\mu\text{M}$  of ribozymes as described in Materials and Methods. In this HeLa X1/5 cell line, the promoter is silent in the presence of tetracycline (34). This property was used to induce the expression of luciferase after transfection by reducing the amount of tetracycline from 100 to 5 ng/ml in the culture medium. Luciferase activity was determined after 18 h of growth as RLU/mg protein. Luciferase activity could not be detected for uninduced cells. Activity in the induced cells varied between  $0.56 \times 10^6$  and  $2.20 \times 10^6$  RLU/mg protein with different cell preparations. Results of two typical experiments with cells with  $1.15 \times 10^6$  RLU/mg protein are presented in Figure 3. Activity with 1  $\mu\text{M}$  ribozymes, using the



**Figure 5.** Northern blot analyses of intracellular ribozymes. HeLa X1/5 cells were transfected with ribozyme-Tfx-50 complexes for 2.5 h. After PBS washing, cells were grown for the indicated time. Total RNA or cytoplasmic and nuclear RNA were isolated from cells and subjected to northern blot analysis as described in Materials and Methods. (A) Blot of total RNA. Times, after transfection; untransfected cells, no Rz. (B) Blot of cytoplasmic (C) and nuclear (N) RNA 24 h after transfection. Upper panels,  $\beta$ -actin RNA; lower panels, ribozymes.

inverted forms as control for the sequence specificity of the inhibitory effect, was reduced to 46% with R865, to 52% with the inactive ribozyme (G5A), to 49% with Rz865M-PS and to 54% with the inactive version (G5A). Error bars indicate the percent activity obtained with two more cell preparations (Fig. 3). Inhibition with the inactive ribozymes with the mutation G5A differed only very little from that obtained with the active forms. Another ribozyme containing two mutations (G5A, A14U) showed 60% inhibition, a value comparable to the G5A ribozyme mutant. No difference was observed by comparing the results of experiments conducted in the absence or the presence of serum during transfection (data not shown). Cells transfected with up to a concentration of 3  $\mu$ M with the cholesterol-modified ribozyme (Rz5865Ch) without cationic lipids, did not show any inhibitory effect on gene expression.

In order to confirm that the decrease of luciferase activity correlated with a decrease in the protein level in the cells, a western immunoblot was prepared by using the same cell extract to determine luciferase activity and the level of luciferase protein (Fig. 4). A good correlation was found between inhibition and reduction of the protein level.

The effect of ribozyme was followed up to 30 h. At this time inhibition was still observed for both ribozymes Rz865 and

Rz865M-PS (data not shown). Measurements over a period of longer than 30 h were not possible because continual cell growth led to increasing cell density, resulting in a high degree of cell death interfering with the expression data of the luciferase.

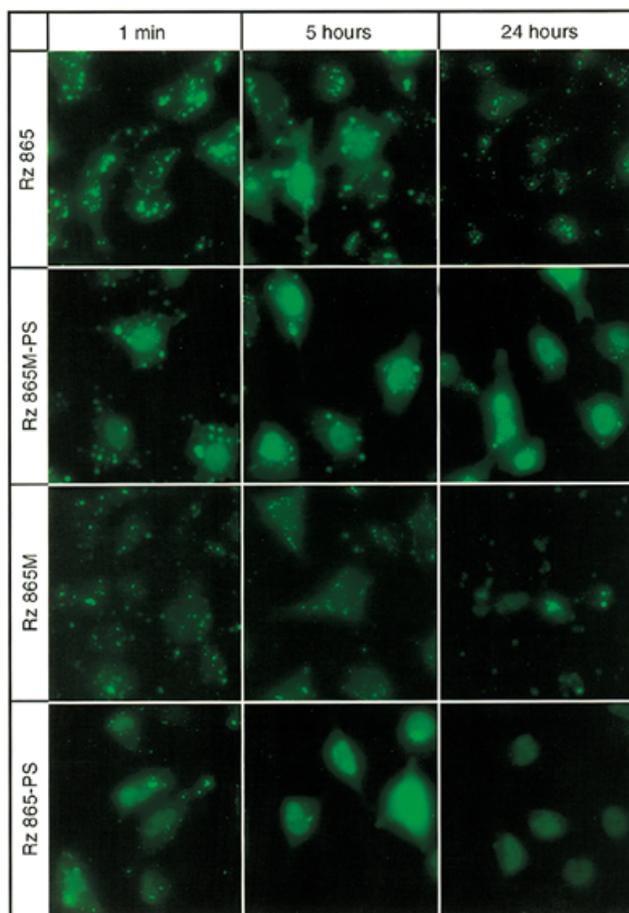
#### Cellular uptake of ribozymes and stability in cells

Analysis of ribozymes in the cells was obtained by northern blots (Fig. 5). Surprisingly, the blotting efficiency varied with the chemical modification of the ribozymes. In separate experiments, concentrations ranging from 17 to 48 pmol of each ribozyme in control blots, and setting the value with Rz865 as 100%, the blotting efficiency for Rz865M was 75% and that for Rz865M-PS was 49%. Total RNA was first probed for ribozymes and then for actin to normalise the ribozyme blotting values for the amount of cellular RNA. The amount of ribozymes 1 min after transfection were 114 pmol for Rz865 as an average of two experiments, 120 pmol for Rz865M and 114 pmol for Rz865M-PS in 10  $\mu$ g of total RNA (Fig. 5A). These values represent an average of two experiments.

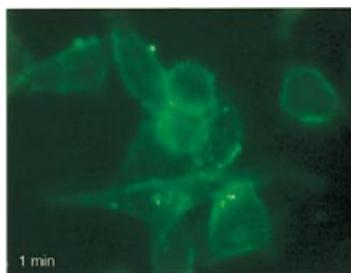
For the determination of the distribution of ribozymes in the cytoplasm and in the nucleus, the actin blot was used as a measure for the carry-over of cytoplasmic RNA to the nuclear RNA (Fig. 5B). The ratio of ribozymes in the two fractions was corrected against the actin values. After 24 h of transfection, Ribozyme Rz865 was localised in the cytoplasm to an extent of 66% (13 pmol) and up to 34% (7 pmol) in the nucleus. The corresponding values for Rz865M were 67 and 33%, and for Rz865M-PS were 43% (18 pmol) and 57% (23 pmol), respectively.

Cellular uptake of fluorescein-labelled ribozymes was followed by fluorescence microscopy. The uptake properties were compared for ribozymes Rz865, Rz865M, Rz865PS and Rz865M-PS either with or without complexation to Tfx-50. Transfected cells were incubated at 37°C for times ranging from 0 to 24 h. The intracellular localisation of ribozymes at various time-points, after transfection, was immediately documented with living cells to avoid artefacts introduced by fixation procedures (Fig. 6). One minute after ribozyme transfection, no fluorescence was observed in cells transfected in the absence of Tfx-50, indicating a weak or no cellular uptake (data not shown). A speckled fluorescence pattern in the cytoplasm was observed for the unmodified ribozyme (Rz865) 1 min after completion of transfection, aided by lipofection, which then became more regular after 5 h with concentration in the nucleus in a few cells. Fluorescence, although still detectable, became very weak after 24 h. Cells transfected with Rz865M-PS showed a more regular fluorescence with high concentration in the nucleus over the whole time period. Cells transfected with ribozyme Rz865M, with the same 2'-modifications as Rz865M-PS but without the phosphorothioate linkage, showed a fluorescence pattern over time resembling closely to that found in cells transfected with Rz865 (Fig. 6). Rz865-PS, which carries phosphorothioates as the sole modification, was also predominantly localised in the nucleus. This pattern suggests that phosphorothioates influence the localisation of the ribozyme in the cell.

Additionally, the uptake of the cholesterol-modified ribozyme Rz865Ch was investigated in the absence of Tfx-50. Subcellular fluorescence patterns of these cells were different (Fig. 7). One minute after transfection, the distribution of ribozymes in cytoplasm was more diffuse without punctated

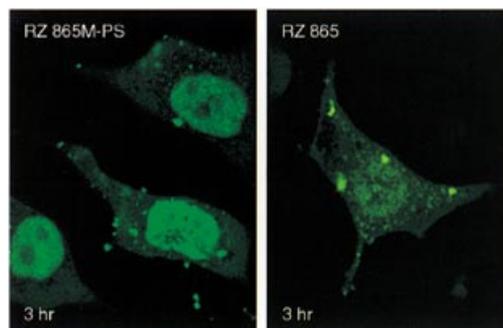


**Figure 6.** Fluorescence microscopy of cellular uptake of ribozymes. HeLa X1/5 cells were incubated with fluorescein-labelled ribozymes (Rz865, Rz865M, Rz865M-PS, Rz865-PS) complexed to Tfx-50 for 2.5 h. After PBS washing, cells were grown and living cells were subjected to fluorescence microscopy at indicated times using a 100 $\times$  objective.

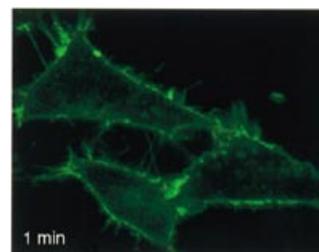


**Figure 7.** Fluorescence microscopy for the cellular localisation of 5'-fluorescein-labelled cholesterol ribozymes (Rz865Ch). Cells (HeLa X1/5) were treated with the ribozyme for 2.5 h. After PBS washing, living cells were subjected to fluorescence microscopy using a 100 $\times$  objective. Time after transfection is indicated.

structures and more fluorescence intensity was observed near the membrane area. No difference in fluorescence intensity between cytoplasm and nucleus was observed. It was found that 6 h post-transfection, the fluorescence had almost disappeared (data not shown). 2'-Modifications of the cholesterol-



**Figure 8.** Confocal laser scanning microscopy of the intracellular distribution of 5'-fluorescein-labelled ribozymes. HeLa X1/5 cells treated with 2'-modified ribozymes (Rz865M-PS) and with the unmodified ribozymes (Rz865). Cells were treated with ribozyme-Tfx-50 complexes for 2.5 h. After PBS washing, cells were incubated for 3 h. Living cells were subjected to laser-assisted confocal microscopy (magnification: 200 $\times$ ).



**Figure 9.** Confocal laser scanning microscopy of the cellular uptake of 5'-fluorescein-labelled cholesterol ribozymes (Rz865Ch). Cells were incubated with the ribozyme for 2.5 h. After PBS washing, living cells were subjected directly to laser assisted confocal microscopy (magnification: 200 $\times$ ).

conjugated ribozyme did not change the distribution (data not shown).

In order to confirm the localisation of the ribozymes in the cytoplasm and the nucleus and to discriminate between cell surface binding and intracellular localisation, cellular uptake was also studied by CLSM, a method that allows an optical section to be cut through the cell. This analysis confirmed the results of cellular uptake of the ribozymes by fluorescence microscopy (Fig. 8). Three hours after transfection, cells treated with Rz865M-PS showed homogenous intracellular fluorescence with a much higher intensity in the nucleus than in the cytoplasm. In contrast, cells transfected with the unmodified ribozyme (Rz865) showed a much weaker fluorescence intensity with similar distribution between cytoplasm and nucleus.

In case of cells transfected with cholesterol-modified ribozymes, fluorescence demonstrated that ribozymes were predominantly localised in the cell membrane with only slight intracellular fluorescence (Fig. 9).

## DISCUSSION

Two major obstacles are associated with the exogenous delivery of synthetic ribozymes to cells for the inhibition of

gene expression (1–3). These are the instability of ribozymes against nucleases and their inefficient cellular uptake. To address these questions cell culture experiments were performed with a cell line which stably expresses luciferase under control of a tetracycline-regulated promoter (34). This system was chosen to control the onset of mRNA synthesis and to determine the effect of ribozymes as a function of mRNA production. Unmodified and 2'-modified ribozymes were used for these studies to investigate advantages of chemically stabilised ribozymes as potential therapeutic agents *in vivo*. Additionally, cellular uptake was investigated with these ribozymes in the presence or absence of a cationic lipid. A ribozyme bearing a cholesterol modification at the 3' end was also analysed in order to facilitate direct transfection of cells.

A number of cationic lipids have been successfully used for exogenous delivery of oligonucleotides in cell culture (23–25). Most transfection agents possess some cell toxicity and thus, can only be used in the absence of serum during the transfection procedure. Tfx-50 was found to be a satisfactory low-toxic transfection agent for our system that facilitated efficient transfection of cells even in the presence of serum. However, no difference in cellular uptake and in the efficiency of inhibition of gene expression was observed in the absence or the presence of serum.

Ribozymes Rz865 and Rz865M-PS showed inhibition of luciferase gene expression in the cell culture experiments when used with Tfx-50. Inhibition of gene expression was not observed without the use of this agent. The degree of inhibition was ~50% (Fig. 3). The reduction in activity was also matched with a reduction in protein levels as shown by a western blot (Fig. 4). Interestingly, the degree of inhibition was almost identical for the active and for the inactive ribozyme with a G5A mutation (Fig. 3) and another with two mutations (G5A and A14U). The inhibition of luciferase expression by the two inactivated ribozymes argues for an antisense effect being responsible for the inhibition in our system. However, there are reports that the inhibition was more pronounced with the active ribozyme than with the inactive one when applied exogenously (4–8,11).

We attempted to correlate ribozyme inhibition with their cellular uptake by northern blot analysis (Fig. 5). Unmodified and modified ribozymes were detected in cells in similar amounts. Surprisingly, the reduction of ribozyme concentration, 24 h after transfection, was comparable for all ribozymes even though Rz865M-PS should be particularly stable to degradation by nucleases. The ratio of distribution of ribozymes between the cytoplasm and the nucleus was two for the ribozymes lacking the phosphorothioate groups but 0.75 for Rz865M-PS, indicating predominant localisation in the nucleus.

For a more detailed picture of cellular localisation, fluorescently-labelled ribozymes were used for microscopic visualisation. Strong fluorescence observed in this study confirmed the northern blot results that modified and unmodified ribozymes were taken up by cells efficiently when complexed to Tfx-50. The observed punctated fluorescence pattern indicates that ribozymes are compartmentalised (Fig. 7). This is in agreement with other reports that the complexes of cationic lipids and synthetic ribozymes (40,41), as well as those with antisense-oligodeoxynucleotides (25,42) are internalised by endocytosis, and the trapping in endosomes explains the punctuated picture.

The fluorescence pattern as a function of time was clearly different for Rz865 and Rz865M compared to Rz865M-PS. The vesicular localisation in the cytoplasm changed rapidly to regular cytoplasmic and enhanced nuclear accumulation for Rz865M-PS, whereas the punctated structures did not change for Rz865 as well as for Rz865M, both lacking the phosphorothioates. Only weak nuclear and cytoplasmic accumulation was observed for Rz865 and Rz865M. After 24 h, Rz865M-PS fluoresced more intensely in the cells compared to Rz865 and Rz865M. A distribution similar to Rz865M-PS was also observed for Rz865-PS.

Confocal microscopy was employed to further analyse the localisation of the ribozymes in the cell. It confirmed that ribozymes were localised in the cell (Fig. 8). Cells transfected with the modified ribozyme Rz865M-PS showed a very strong fluorescence in the nucleus compared to those transfected with ribozymes without phosphorothioate linkages (Rz865) where fluorescence was weaker in general and not concentrated in the nucleus.

These findings from the fluorescence are in agreement with several other reports analysing cellular uptake and distribution of oligodeoxynucleotides or ribozymes transfected via lipofection. A regular fluorescence in cells with an intensely fluorescent nucleus was observed when transfection was carried out with oligodeoxynucleotides bearing phosphorothioate linkages (25,42–44) and ribozymes with phosphorothioates (10,13). On the other hand, a punctated fluorescence pattern in the cytoplasm was described for oligodeoxynucleotides and for ribozymes without phosphorothioate linkages, independent of other modifications (10,41,43,45). Thus, as shown here, the presence of phosphorothioates seems to be responsible for the localisation of the ribozymes in the nucleus.

The northern blot indicates twice the amount of Rz865 in the cytoplasm than in the nucleus. Considering that the volume of the cytoplasm is considerably larger than that of the nucleus, the fluorescence of similar intensities in these two compartments would agree with that distribution. More of Rz865M-PS in the nucleus than in the cytoplasm would be consistent with high fluorescence in the former even though the strength of the fluorescence signal seems quite strong for the amount of ribozyme in the nucleus.

Both ribozymes Rz865 and Rz865M-PS had the same inhibitory effect. The amount of both ribozymes in the cytoplasm is very similar, but three times higher in the nucleus for Rz865M-PS. This suggests that the ribozymes in the cytoplasm are primarily responsible for the inhibition. If ribozymes in the nucleus contributed significantly, one would expect Rz865M-PS to have a higher inhibitory activity.

There is precedent for the interpretation that ribozymes in the cytoplasm are responsible for the inhibition of gene expression. Synthetic, chemically-modified ribozymes inhibited HIV-1 replication when microinjected into the cytoplasm but not in the nucleus (46). Similarly, inhibition of HIV-1 replication by ribozymes applied endogenously was observed when the ribozymes were localised in the cytoplasm (47). Activity of cytoplasmic as opposed to nuclear ribozymes had also been demonstrated in the transgenic zebrafish (48).

It was expected that Rz865M-PS might inhibit luciferase expression more strongly and for a longer period than Rz865 because of its nuclease stability. However, both ribozymes were equally effective. Apparently, as this has been observed

previously, the lipofection agent confers protection to an unmodified ribozyme against nuclease degradation, even in serum-complemented medium (8,11). Once inside the cell, degradation seems to be a slow process (43).

Previous reports had indicated that oligodeoxynucleotides could be taken up and internalised by cultured cells, without lipids, if they were conjugated to a cholesterol moiety (28–33). This improved uptake was attributed to an increase in absorption of the oligonucleotides via hydrophobic interactions with the cell membrane or mediated via the LDL receptor. Thus, it was expected that ribozymes might follow a similar path of uptake. Rz865Ch was constructed to take advantage from reducing conditions in the cell to cleave the disulfide bridge connecting cholesterol and the ribozyme, to release the ribozyme. Even though this ribozyme showed cleavage of the transcript *in vitro*, no reduction in luciferase activity could be discerned in the cell culture system. The confocal microscopic analysis clearly demonstrated that this ribozyme predominantly remained trapped in the cell membrane, preventing interaction with the mRNA in the cytoplasm (Fig. 9). The question remains why these results differ from those reported for oligodeoxynucleotides. One reason could be that the LDL receptor-mediated uptake was not accessible in our system as we applied the cholesterol-ribozyme in the absence of serum. Another possible explanation could be a size effect, as Rz865Ch is almost twice as long as conventional antisense oligonucleotides. Additionally, it has been shown that the type of linker between an antisense oligonucleotide and the cholesterol can also influence efficacy of inhibition (33).

The results obtained here and those in another report (8) indicate that the advantages of chemically-stabilised ribozymes for exogenous cellular delivery are not obvious. These findings are also supported by other reports where unmodified ribozymes were successfully used in cell culture experiments (16,17,19). However, a comparison of exogenously delivered 2'-modified ribozymes with unmodified ribozymes in animal model studies showed that only the former were active (20,21). In these studies no cationic lipids or other carriers had to be used for delivery, indicating a fundamentally different uptake mechanism of oligonucleotides in cell culture and animals.

This study shows that despite published data on the success of exogenously delivered ribozymes for inhibition of gene expression, the method cannot yet be considered general. Although we observe inhibition, cleavage activity of the ribozymes does not make a significant contribution in our system. Further, the fluorescence microscopy results seem to support the notion that cytoplasmic localisation is important and should be aimed for in future studies. The inefficient uptake of the cholesterol-modified ribozymes, in contrast to that of oligodeoxynucleotides, concerns another area for future research.

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