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# *mdr*1 Ribozyme mediated reversal of the multi-drug resistant phenotype in human lung cell lines

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**Abstract**

An *mdr*1 hammerhead was introduced into two adriamycin-selected multi-drug resistant human lung cell lines both of which over-express p-glycoprotein. Expression of the ribozyme resulted in a decrease in *mdr*1 mRNA expression and an increase in drug sensitivity in both cell lines. This would suggest that the use of specific ribozymes may represent an effective and specific approach in order to restore cellular sensitivity towards anti-cancer drugs.

## Introduction

The resistance of tumour cells to chemotherapeutic drugs is a major limitation in cancer treatment (Teicher, 1993; Clynes, 1994). Many tumours are intrinsically resistant to the most potent cytotoxic agents used in cancer therapy; other tumours, initially sensitive to a particular chemotherapeutic regime, become progressively un-responsive (acquired resistance), not only to the initial therapeutic agent but also to other drugs to which the tumour has not previously been exposed (cross-resistance). Although several potential mechanisms have been proposed to explain this phenomenon, increased expression (due to gene amplification and/or over-expression of *mdr-1* mRNA) of p-glycoprotein (p-gp) has been the most extensively characterized (Endicott and Ling, 1989; Schinkel *et al.*, 1991). Over-expression of p-gp is accompanied by the enhanced synthesis of the 4.5 kb *mdr*1 mRNA which codes for p-gp (Roninson *et al.*, 1986). P-gp is a 170 kDa transmembrane protein that functions as an energy-dependent efflux pump which prevents the intracellular accumulation of drugs reaching an effective cytotoxic concentration (Endicott and Ling, 1989).

Ribozymes are catalytic antisense RNAs that function as enzymes capable of site-specific cleavage of substrate RNA in a sequence-dependent manner (Cech and Bass, 1986). Ribozymes contain stretches of nucleotides that basepair with complementary RNA regions and have a catalytic section, analogous to the active site of a protein enzyme, that cleaves the bound RNA while the basepairs hold it in place (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Cameron and Jennings, 1989). Hammerhead ribozymes can cleave the 3′ end of the triplet GUX (where X is C, A or U) in RNA molecules. As ribozymes can destroy a specific RNA by cutting it in two they can potentially be used for the selective inhibition of a disease-related protein (Scanlon *et al.*, 1991; Funato *et al.*, 1992; Kashani Sabet *et al.*, 1992; Holm *et al.*, 1994; Scanlon *et al.*, 1994) or as potential anti-viral agents (Sarver *et al.*, 1990).

In this paper we describe the use of a hammerhead ribozyme targeted against *mdr*1 mRNA. The ribozyme was introduced into two adriamycin-resistant lung cell lines to evaluate its influence on the MDR phenotype of these cell lines. The ribozyme used was designed to cleave the GUC sequence at codon 880 of exon 21 of *mdr*1 mRNA (See Figure 1 in Holm *et al.*, 1994). This target site resides between two ATP-binding sites, with possibly important implications for p-gp function (Gottesmann and Pastan, 1993). The ribozyme

*Table I.* $IC_{50}$ values obtained for a range of chemotherapeutic drugs for cell lines used in the *mdr*1 ribozyme studies. The values given are the mean $IC_{50}$ value (4 experiments)

| $IC_{50}$ (nM) | Adriamycin | Vincristine | VP-16 |
|---|---|---|---|
| DLKP | 24.8 | 1.21 | 146.45 |
| DLKP-A | 6310 | 1820.1 | 8885.5 |
| SKMES-1 | 41 | 2.54 | 567.7 |
| SKMES-1ADR | 465.8 | 265.5 | 3841.8 |

*Table IA.* Fold resistance levels of cell lines used in the *mdr*1 studies

| Fold resistance | DLKP-A: DLKP | SKMES-1ADR: SKMES |
|---|---|---|
| Adriamycin | 254 | 11.4 |
| Vincristine | 1504 | 104.5 |
| VP-16 | 61 | 6.8 |

was demonstrated to cleave *in vitro* a target 240 base RNA substrate into 138 and 102 basepair products, while the 43 basepair ribozyme remained unaffected (Holm *et al.*, 1994). The *mdr*1 ribozyme was cloned into the eukaryotic expression vector pH$\beta$APR-1neo as described by Holm *et al.* (1994). This ribozyme's ability to cleave *mdr*1 mRNA in transfected cell lines has been demonstrated by Scanlon *et al.* (1994); they describe the production of two cleaved products of the expected size as analysed by Northern analysis.

## Materials and methods

### *Cell lines*

The drug-sensitive human lung squamous cell carcinoma line DLKP was established from a tumour biopsy in this laboratory (Law *et al.*, 1992). The DLKP-A resistant cell line was isolated by step-wise selection in increasing concentrations of adriamycin (Clynes *et al.*, 1992). $IC_{50s}$ of this cell line are given in Table I, while Table IA contains fold resistance levels. Resistance in this line is stable for at least three months, in culture, in the absence of drug.

SKMES-1 is a human lung squamous carcinoma cell line (ATCC No. HTB 58). SKMES-1ADR was derived from the cell line SKMES-1 by step-wise exposure to adriamycin. For $IC_{50}$ and fold resistance levels see Tables I and IA.

The drug sensitive DLKP and its drug resistant variant DLKP-A were grown in DMEM/HAMsF12 supplemented with 5% Fetal Calf Serum and 20 mM Hepes (Sigma H9139). SKMES-1 and SKMES-1ADR were grown in MEM supplemented with 5% fetal Calf Serum.

### *Toxicity assay to assess $IC_{50}$ values*

As a functional assay to determine drug resistance the cell lines and selected clones were subjected to a toxicity assay and stained using Crystal Violet as described by Martin and Clynes (1993). The $IC_{50}$ is the concentration that inhibits cell growth by 50%. Cells were plated from a single cell suspension into a 96 well plate (Costar, 3599) at a cell density of $10^3$ cells/well in 100 $\mu$l of medium. The first column of the plate was used as a plate control and contained only medium. Cells were allowed to attach for 24 hours at 37 °C and 5% $CO_2$. The required drug dilution (100 $\mu$l) (which was diluted to 2× the final drug concentration required in the assay) was added to each well in replicas of 8. Drug concentrations used in each assay ranges from zero, to a concentration which achieved approximately 100% cell kill. The plates were then incubated for a further 6 days at 5% $CO_2$ and 37 °C or until control wells (i.e. no drug added) reached 80% confluency. Drug toxicity was then determined by Crystal Violet staining. After removal of medium the 96 well plates were rinsed with 100 $\mu$l of PBS/well and stained with 100 $\mu$l of 0.25% (w/v) aqueous Crystal Violet (prefiltered with Whatman No 1 paper) for 10 mins. Plates were rinsed four times in tap water and allowed to dry at 37 °C. When dry 100 $\mu$l/well of 33% glacial acetic acid (BDH 10001) was added and the contents of each well mixed before reading at 570 nm on a Titertek Multiskan-plus ELISA plate reader using 620 nm as the reference wavelength.

### *Plasmid construction*

The *mdr*1 ribozyme was prepared from two synthetic single-stranded oligodeoxynucleotides as described by Kashani-Sabet *et al.* (1992) and was cloned into the eukaryotic expression vector pH$\beta$APr-1neo (Gunning *et al.*, 1987) which contains a $\beta$-actin promoter sequence and a gene for neomycin selection. The ribozyme containing construct was termed pH$\beta$APR-1*mdr*rz.

*Transfection*

Transfection of cell lines with the plasmid pH$\beta$APR-1*mdr*1z was carried out using a standard calcium phosphate precipitation (Sambrook *et al.*, 1989). Briefly, cells to be transfected were plated at a density of $1\times10^5$ – $2\times10^5$ cells/cm$^2$ in a 60 mm tissue culture dish in the appropriate medium. Cells were allowed to attach for 24 hours at 37 °C in a humidified incubator in an atmosphere of 5% $CO_2$. A calcium phosphate/DNA co-precipitate was prepared as follows: 220 $\mu$l of DNA (20 $\mu$g/$10^6$ cells in 100 mM TE pH 8.0 at a concentration of 40 $\mu$g/ml) and 250 $\mu$l of 2×HBS was mixed in a disposable sterile 5 ml plastic tube. 2 M $CaCl_2$ (31 $\mu$l) was slowly added with gentle mixing over a period of approximately 30 seconds. The mixture was then incubated for 20–30 minutes at room temperature during which time a fine precipitate formed. At the end of the incubation the mixture was pipetted up and down to resuspend the precipitate. The cells were removed from the incubator and the DNA-$CaPO_4$ mix was added dropwise to the cells while swirling the plate gently to ensure even mixing. The cells were then incubated at 37 °C for 4 hours. In order to increase the efficiency of transformation the cells were glycerol-shocked. The media containing the DNA was removed from the cells and 5 ml of 10% glycerol in 1×TBS was added. The cells were then incubated for 3 minutes, the glycerol removed and the cells washed once with PBS. The cells were re-fed with 10 ml of fresh growth media. The plates were incubated for 24–60 hours at 37 °C before replating the cells in the appropriate selective medium for the isolation of stable transformants. Transfection of the plasmid pH$\beta$APr-1neo which does not contain the ribozyme sequence served as a negative control. This excludes the possibility that reversal of the MDR phenotype might be a consequence of plasmid incorporation. Transfected cells were propagated in medium containing geneticin (500 $\mu$g/ml) G418 sulphate (GIBCO) for 4 weeks. Individual G418 resistant colonies were isolated, grown and screened for expression of the *mdr*1 ribozyme.

*RT-PCR analysis*

RT-PCR was used to detect ribozyme expression (Kashani Sabet *et al.*, 1992) and to analyse *mdr*1 expression (O'Driscoll *et al.*, 1994) in the chosen clones. PolyA (1 $\mu$g) from the cell lines was used to synthesise cDNA. The reverse transcriptase reaction was carried out as follows; oligo-dT primers (1 $\mu$g/$\mu$l) (1 $\mu$l,) 1 $\mu$g of polyA and 2 $\mu$l of water were combined and incubated at 70 °C for 10 minutes. The reaction was then chilled on ice. To this reaction was added 50 mM Tris HCl pH 8.3, 75mM KC1, 3 mM $MgCl_2$, 10 mM DTT, 2 units RNAsin, 0.5 mM dNTPs, 10 units of MMLV-RT and the volume made up to 20 $\mu$l with water. The reaction was then incubated at 37 °C for 1 hour, followed by 95 °C for 2 mins.

A portion (5 $\mu$l) of this reaction was then amplified as follows: 10 mM Tris HCl pH 9.0, 5 mM KCl, 0.1% Triton X 100, 2.5 mM $MgCl_2$, 0.2 mM sNTPs, 250 ng of the required primers and 5 units of Taq DNA polymerase was added to each tube. A drop of mineral oil was added to each reaction and PCR amplification carried out according to the appropriate conditions for the particular set of primers being used.

*RNA Isolation*

PolyA mRNA was isolated using a modification of the method outlined in Sambrook *et al.*, 1989. The culture media was poured from the flasks and the attached cells washed twice with DEPC treated PBS. 5 ml of lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 2 mM EDTA, 1% SDS) per $10^6$ cells was added to the cells. The lysed cells were collected in a sterile tube and 50-100 $\mu$g/ml Proteinase K was added to the solution. The mixture was incubated at 37 °C for 30 mins. Contaminating DNA was sheared by passing the solution through a syringe and 0.22 mm needle. At this point the solution can be stored at –80 °C before being processed further. Proteinase K (50 $\mu$g/ml) was added and the solution incubated at 37 °C for 30 mins. NaCl to 0.4 M was added and the complete mix transferred to a sterile tube containing 10 ml of pre-washed oligo-dT cellulose. The reaction was mixed thoroughly and rocked for 2 hours at room temperature. This solution was centrifuged for 5 minutes and the supernatent discarded. The oligo-dT was washed with 20 ml of high salt buffer (HSB) (10 mM Tris pH 7.4, 0.3 M NaCl, 0.1 mM EDTA, 0.2% SDS), resuspended in 10 ml of HSB, and poured into a column. The packed column was washed three times with 5 ml of HSB and twice with 1 ml of low salt buffer (10 mM Tris 7.4, 0.1 M NaCl, 0.1 mM EDTA, 0.2% SDS). PolyA was eluted from the column by washing it with three 500 $\mu$l washes of no salt buffer (5 mM Tris pH 7.4, 0.1 mM EDTA, 0.2% SDS). These fractions were collected and pooled; the entire mix was brought to 0.3 M sodium acetate and 2 volumes of cold ethanol added. mRNA was allowed to precipitate overnight at –20 °C.

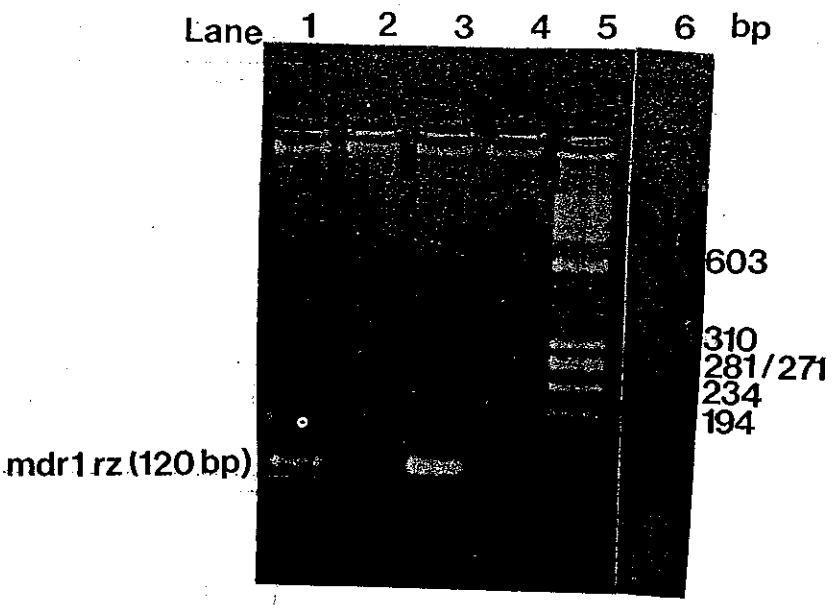


*Figure 1.* Detection of *mdr*1 ribozyme expression in transfected clones of DLKP-A. The gel shows the PCR products amplified from 1 $\mu$g of polyA mRNA from clone 1 (lane 1), clone 2 (lane 2), clone 3 (lane 3) and untransfected DLKP-A (lane 4) using the primers shown in Figure 2A. Lane 5 contains a DNA molecular weight marker. Lane 6 contains the water control. Amplification of ribozyme mRNA results in the production of a 120 base spliced ribozyme product.

Rib1: 5′ AGC ACA GAG CCT CTT T 3′

Rib2: 5′ TCT CGA TCC CTC GAA GC 3′

Fig. 2A. Primers for RT-PCT analysis of *mdr*1 ribozyme expression. Rib1 is complementary to a region of the $\beta$-actin promotor in the plasmid pH$\beta$APR-1neo while Rib2 binds to a region in the polylinker of the plasmid. Using these two primers a 120 basepair spliced ribozyme product is amplified. If the expression vector pH$\beta$APr-1*mdr*rz serves as a template for PCR a 950 basepair DNA band is amplified.

## Results

Cell lines were transfected with the pH$\beta$Apr-1*mdr*rz plasmid by calcium phosphate precipitation and a number of clones which survived selection in G418 were isolated. Stable expression of the ribozyme was demonstrated using RT-PCR (Figure 1). Using the two primers Rib 1 (which is complementary to a region downstream of the transcription initiation site in the $\beta$-actin promoter (Gunning *et al.*, 1987) in the plasmid pH$\beta$APr-1*mdr*rz) and Rib 2 (which is complementary to a region in the polylinker of the plasmid) (for sequence of primers see Figure 2A) a spliced ribozyme product of 120 basepairs was amplified from ribozyme mRNA. If the expression vector pH$\beta$APR*mdr*rz plasmid alone served as a template for PCR a 950 basepair product is amplified.

Both Northern Blot analysis and RT-PCR were used to study the expression of *mdr*1 in the ribozyme containing clones. Overexpression of *mdr*1 mRNA in the drug resistant DLKP-A cell line is evident. In the ribozyme transfectants of the MDR cell line DLKP-A and SKMES-1ADR the levels of *mdr*1 mRNA were reduced (Figure 3). Similar results were obtained for the SKMES-1ADR line and its ribozyme-containing clones (results not shown).

The clones from each cell line which were shown to have the highest levels of *mdr*1 ribozyme expression were chosen for further analysis. Toxicity assays were carried out on these clones and cells stained using Crystal Violet.

No significant change in drug resistance in ribozyme transfectants of the parental drug sensitive DLKP and SKMES-1 cell lines was evident. A

Sense: 5′ CCC ATC ATT GCA ATA 0GCA GG 3′

Antisense: 5′ GTT CAA ACT TCT GCT CCT GA 3′

Fig. 2B. Primers for RT-PCR analysis of *mdr*1 RNA expression. The sense primer is located from position 2596 to 2615 on the *mdr*1 template while the antisense primer is located from position 2733 to 2752 (Noonan *et al.*, 1990). PCR amplification of *mdr*1 cDNA using these primers result in the amplification of a 157 basepair product.

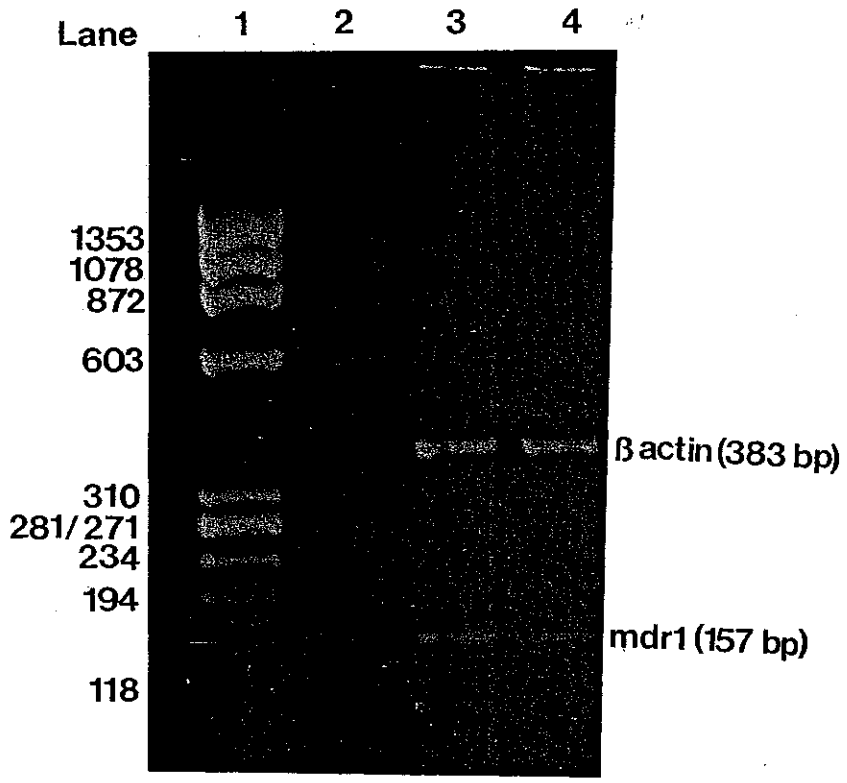


*Figure 3.* Gene expression of *mdr*1 in *mdr*1 ribozyme containing clones as assayed by RT-PCR. The gel shows the amplified PCR products generated from 1 $\mu$g of polyA mRNA from DLKP-A parent (lane 3) and clone 3 (lane 4). For the sequence of the *mdr*1-specific primers used see Figure 2B. Lane 1 contains molecular weight markers while lane 2 contains the water control. $\beta$-actin was amplified as an internal control using specific primers to amplify a band of 383 base pairs.

*Table 2.* $IC_{50}$ (nM) levels of SKMES-1ADR and DLKP-A cell lines and a number of *mdr*1 ribozyme clones

| $IC_{50}$ (nM) | Adriamycin | Vincristine | VP-16 |
|---|---|---|---|
| DLKP-A | 6310 | 1820.1 | 8885.5 |
| DLKP-A*mdr*rz clone 1 | 1577 | 67 | 2538.7 |
| DLKP-A*mdr*rz clone 2 | 1856 | 98 | 3702 |
| SKMES-1ADR | 465.8 | 265.5 | 3841.8 |
| SKMES-1ADR*mdr*rz clone 1 | 203.4 | 157.3 | 2745 |
| SKMES-1ADR*mdr*rz clone 2 | 184.11 | 181.2 | 2170.4 |

decrease in drug resistance was evident in clones of the DLKP-A and SKMES-1ADR cell lines containing the ribozyme (see Table 2). In the SKMES-1ADR *mdr*1 ribozyme clones the level of resistance to adriamycin and VP-16 was reduced by up to 3-fold and 2-fold respectively, while resistance to vincristine was reduced by 2-fold. In the ribozyme transfectants of the DLKP-A cell line the greatest effect was evident in the level of vincristine resistance (up to 25-fold reduction); adriamycin and VP-16 resistance levels were reduced by 4 and 3.5-fold respecctively.

## Discussion

In this study we attempted to examine the effect of an *mdr*1 ribozyme on two MDR lung cell lines. The drug-sensitive human lung squamous cell carcinoma line DLKP was established from a tumour biopsy in this laboratory (Law *et al.*, 1992). The DLKP-A resistant cell line was isolated by stepwise selection in increasing concentrations of adriamycin (Clynes *et al.*, 1992). Previous work on this lung line using *mdr*1 antisense oligonucleotides (Clynes *et al.*, 1992) and circumvention agents specific for p-gp (e.g. verapamil) have indicated that p-gp may not explain the entire mechanism of resistance in this line; other as yet unidentified mechanisms of resistance would appear to account for part of the resistance profile (Cleary, 1995). DLKP-A is a heterogenous population of cells in terms of drug resistance which reflects the situation in tumours in the body. In particular lung tumours have been found to possess a large degree of tumour heterogeneity (Heppner, 1984). The cell line SKMES-1ADR (derived from the cell SKMES-1 by step-wise exposure to adriamycin) is less well characterised though p-gp is believed to contribute to its mechanism of resistance (Cleary and Clynes, unpublished).

A plasmid containing the *mdr*1 ribozyme (pH$\beta$APr-1*mdrrz*) was transfected into both cell lines. This plasmid contains the $\beta$-actin gene promoter and encodes a ribozyme previously demonstrated to cleave the GUC sequence at codon 880 of exon 21 of *mdr*1 mRNA (Holm *et al.*, 1994). Clones that express the ribozyme were isolated from each cell line transfected.

Resistance in the ribozyme-containing transfectants of the drug resistant DLKP-A cell line was reversed 4-fold to adriamycin and to vincristine by up to 27-fold. These results compare favourably with previously reported studies using *mdr*1 ribozymes; Scanlon *et al.* (1994) reported a reversal of adriamycin resistance in a drug resistant human ovarian carcinoma cell line of 2.4-fold and of vincristine resistance of 14.8-fold, while Bertram *et al.* (1995), using a drug resistant human adenocarcinoma line, reported a reduction in $IC_{50}$ levels of 50%. However, the fold reduction levels obtained in our study are considerably less than those described by Holm *et al.* (1994) using the same ribozyme in a human pancreatic carcinoma line.

In both the SKMES-1ADR and the DLKP-A cell lines reducing the level of *mdr*1 mRNA using the ribozyme does not result in complete reversal of resistance to the level of the sensitive parent. It would appear that the level of *mdr*1 mRNA remaining in the *mdr*1 ribozyme transfectants is capable of mediating drug resistance and/or other mechanisms of resistance may be present. Transfection of the *mdr*1 ribozyme into a number of clones isolated from the DLKP-A cell line each having different levels of resistance has shown that the ribozyme is most effective in reversing resistance in the clone having the lowest level of drug resistance (unpublished studies in this laboratory). In one clone, elimination of *mdr*1 mRNA (as detected using RT-PCR) results in reversal of resistance to almost the level of the drug sensitive parent. This result suggests (as might be expected) that the efficiency of the *mdr*1 ribozyme in reversing resistance is correlated with the level of *mdr*1 expression i.e. the lower the initial level of *mdr*1 gene expression the more efficient the ribozyme is in reversing resistance.

The level of resistance expressed in DLKP-A and SKMES-1ADR is probably higher than would be found in clinical situations, but the results presented here support the idea that ribozymes could be used to alter drug sensitivity in lung tumours.

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