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Upregulation of P-Glycoprotein in Rat Hepatoma ρ° Cells: Implications for Drug–DNA Interactions

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Abstract Rat hepatoma cells lacking mitochondrial DNA (ρ° cells) were used as a model system to examine the possible roles of mitochondrial DNA as a target for the DNA-acting anticancer drug Adriamycin (doxorubicin). The ρ° cells were 45-fold less sensitive to Adriamycin than the parental ρ^+ cells containing mitochondrial DNA. Other non-DNA-acting drugs also exhibited similar behaviour, and this was shown to be due to a multidrug resistance (MDR) phenotype in the ρ° cells. This was indicated by confocal microscopy where ρ^+ cells exhibited thirteenfold higher cellular levels of Adriamycin than ρ° cells. Upregulation (tenfold) of P-glycoprotein in ρ° cells was also confirmed by Northern dot blot analysis. Since the MDR phenotype is present in ρ° cells and upregulation of P-glycoprotein is maintained in these cells, ρ° cells are not a good model system for drug-DNA studies (where the drug is susceptible to extrusion by P-glycoprotein), and any such results obtained with this system must be treated with considerable caution. J. Cell. Biochem. 69:463–469, 1998.

Key words: Adriamycin; rat hepatoma; ρ° cells; multidrug resistance; P-glycoprotein; Sandoz SDZ PSC 833

More than half of the anticancer drugs in current clinical use impair the normal functions of nuclear DNA (nDNA) [Schacter et al., 1992; Ferguson and Pearson, 1996]. However, it is likely that agents which bind to or damage nDNA would also have some capacity to bind to or damage mitochondrial DNA (mtDNA). Indeed, the mutation rate of mtDNA is estimated to be 5–500 times higher than that of nDNA [Allen and Coombs, 1980; Singh et al., 1992]. Lack of a mtDNA protein coat, lack of efficient repair systems in the mitochondrion, and the continuous exposure of mtDNA to oxygen free radicals generated by aerobic metabolism in the mitochondrion are thought to contribute to the elevated mutation rate in mtDNA [Shay and Werbin, 1987; Singh et al., 1992]. It is therefore not surprising that mtDNA is increasingly being recognised as a potential target for a number of anticancer drugs. For example, aflatoxin B_1 [Niranjan et al., 1982], cisplatin [Murata et al., 1990; Olivero et al., 1995, 1997; Giurgiovich et al., 1997a,b], nitric oxide [Wilson et al., 1997], N-methylnitrosourea [Wunderlich et al., 1970; LeDoux et al., 1992], N-nitrosodimethylamine [Wunderlich et al., 1971], 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [Allen and Coombs, 1980; Backer and Weinstein, 1980], and bleomycin [Lim and Neims, 1987] have all been shown to bind preferentially to mtDNA as compared to nDNA. In this study we have used cells lacking mtDNA (ρ° cells) as a model system to explore the concept of mtDNA as a critical target for known DNA-acting drugs. Cells of this phenotype have recently been used as a model system to study the action of non-DNA binding drugs such as a photosensitising boronated porphyrin, which is used in the photodynamic therapy of tumours [Munday et al., 1996]. Adriamycin was selected as a typical, widely used [Devita et al., 1993], and well-characterised [Myers et al., 1988; Weiss, 1992] DNA-acting anticancer agent [Cullinane et al., 1994; Skladanowski and Konopa, 1994; Cutts and Phillips, 1995; Taatjes et al., 1997] for this study and also because it has recently been shown to exhibit mitochondrial toxicity [Kule et al., 1994].

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Mammalian cells can be completely depleted of their mtDNA (ρ° cells) by long-term exposure of actively growing cells to ethidium bromide [King and Attardi, 1989; Herzberg et al., 1993]. Once the ρ° status is induced, ethidium bromide is removed, and the cells canbe maintained in culture in pyruvate and uridine-supplemented growth medium [King and Attardi, 1989]. Using a rat hepatoma (H4) derived ρ° cell line [Martinus et al., 1996], we show by dose-response studies that the sensitivity to Adriamycin in ρ° cells is markedly reduced in comparison to control cells (ρ^+). This difference was shown by RNA dot blot and confocal microscopy to be due to the presence of an upregulated P-glycoprotein phenotype in the rat hepatoma ρ° cells. The use of ρ° cells as a model system to study mtDNA-drug interactions is therefore restricted to those drugs which are not affected by overexpression of P-glycoprotein.

MATERIALS AND METHODS

Materials

Dulbecco's modification of Eagle's medium (DMEM) and the Costar aspiration system were purchased from Trace Biosciences (Castle Hill, NSW). Penicillin/streptomycin solution, foetal calf serum, and TRIzol were obtained from Life Technologies (Bethesda, MD). Pyruvate, uridine, and sulforhodamine B were from Sigma (St. Louis, MO). Tissue culture flasks were purchased from Sarstedt (Adelaide, South Australia). The two-compartment slide chambers and 96-well plates were Nunc products and were supplied by Medos Company (Mt. Waverly, Vic). The haemocytometer was obtained from Selby Scientific (Mulgrave North, Vic). Diethylpyrocarbonate (DEPC) and formamide were from BioScientific (Castle Hill, NSW). Ampicillin was obtained from Boehringer Mannheim (Castle Hill, NSW), while the Qiagen plasmid preparation kit was obtained from Qiagen (Chatsworth, CA). The Rediprime kit, [α-^{32}P]dCTP (Redivue), and nylon transfer membrane (Hybond-N+) were purchased from Amersham (Castle Hill, NSW). G25 Sephadex spin columns were provided by AGP Technology (Mt. Gravatt, Qld), and formaldehyde was purchased from Crown Scientific (Rowville, Vic). Agarose was obtained from Kodak (IBI, CT). Adriamycin was a gift from Farmitalia Carlo Erba (Milan, Italy), while vincristine and taxol were provided by the Peter MacCallum Cancer Institute (Melbourne, Australia) and SDZ PSC 833 was a gift from Sandoz Australia Pty Ltd (Sydney, Australia).

Cell Cultures

A clonal rat hepatoma cell line (H4) (ρ^+) and a mtDNA-less (ρ°) cell line derived from the parent cells [Martinus et al., 1993] were cultured in DMEM supplemented with 45 mM $NaHCO_3$, 1% (v/v) penicillin/streptomycin solution, 1 mM pyruvate, 50 μg/ml uridine, and 10% foetal calf serum. The ρ° cells were passaged 20–30 times prior to use in this study. Adherent cultures of the cells in 25 cm^2 or 75 cm^2 plastic culture flasks were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 in a humidified incubator (Sanyo, Gunma, Tokyo, Japan). Cells were passaged every 2–3 days.

Dose-Response Experiments

Cells were washed with phosphate buffered saline (PBS), harvested by trypsinisation, pelleted at 960 rpm for 5 min, and counted on a haemocytometer (Neubauer type). Cells (approximately (50, 25, 5, 2, and 1×10^3 for one day or less, 2, 3, 5 and 6 day incubations, respectively) were aliquoted into 96-well plates in a volume of 200 μl and allowed to attach for 3 h. Drug was then added to the cells at concentrations ranging from 0-30 μM. After the cells were allowed to incubate for the desired time in the presence of the drug, total protein was assayed by the SRB procedure as described in the next section. The absorbance at 565 nm was plotted as a function of drug concentration using the Origin software package, and the IC_{50} value was calculated as the concentration at which the absorbance was halved.

SRB Assay

The sulforhodamine B (SRB) assay used was a combination of the procedures used by Skehan et al. [1990] and Perez et al. [1993] with minor modifications. After the desired incubation with drug the medium was removed by gentle aspiration and cells were fixed byaddition of 250 μl of cold (4°C) 10% trichloroacetic acid (TCA). The plates were then incubated at 4°C for 1 h, the cells washed five times with Milli-Q water (Millipore, Bedford, MA), and the plates were allowed to air-dry. Cells were then stained with 50 μl of 0.4% SRB in 1% acetic acid for 30 min and subsequently washed five times with 1% acetic acid. The plates were again

allowed to air-dry. The dye was solubilised by the addition of 200 µl of 10 mM Tris (pH 10.5). The plates were then shaken for 5 min on a microplate shaker and the absorbance read immediately using a Molecular Devices (Palo Alto, CA) Spectromax microplate reader at 565 nm.

Confocal Microscopy

Approximately 5×10^5 ρ° or ρ^+ cells were aliquoted into two-compartment slide chambers made for tissue culture and allowed to attach overnight. The multidrug resistance (MDR) reversal agent SDZ PSC 833 (1 µM) was added to the corresponding chambers, and the cells were allowed to incubate at 37°C for 30 min. Adriamycin was then added to the corresponding chambers. The localisation pattern of Adriamycin was detected after 4 h using a Bio-Rad (Richmond, CA) MRC 600 confocal laser scanning microscope fitted to a Nikon (Marunouchi, Tokyo, Japan) diaphot inverted microscope and irradiated with a 10 mW argon-ion laser at 488 nm. Fluorescence emission of 550 nm was selected by a 495DRLP (dichroic long pass) mirror together with a 540LP barrier filter. The confocal analysis was performed using COMOS software (from Bio-Rad) on an IBM 486 computer.

Probing for P-Glycoprotein Levels

Total RNA isolation. Both ρ° and ρ^+ cells were grown to confluency in 175 cm^2 tissue culture flasks, and total cellular RNA was isolated with TRIzol reagent using the Gibco BRL (Life Technologies) protocol supplied with the reagent. The isolated RNA was stored at -70°C in Milli-Q water treated with 0.1% DEPC.

Probe preparation. The plasmid pcDR1.3, containing the P-glycoprotein cDNA insert [Gros et al., 1986], was provided by Dr. Giovani Capronico (National Cancer Institute, Milan, Italy). The plasmid was isolated from a 100 ml plasmid preparation (Luria Bertani medium containing 50 µg/ml ampicillin) according to the Qiagen plasmid purification procedure using Qiagen-tip 100 and stored at 4°C in Tris-EDTA (TE) buffer. The entire pcDR1.3 plasmid was diluted with Milli-Q water (to yield 25 ng of DNA in 45 µl) and the DNA then labelled by random priming with [α-^{32}P]dCTP (3,000 Ci/mmol) using the Amersham Rediprime kit. Unincorporated label and protein were removed with a G25 Sephadex spin column. The eluted probe was then denatured at 95–100°C for 5 min, chilled on ice for 5 min, and then stored at -20°C.

Dot blot analysis. The dot blot analysis was performed using the method of Brown [1991]. Total cellular RNA was denatured using a 66% formamide/21% formaldehyde/13% MOPS denaturing solution and blotted onto a nylon membrane using a dot blot manifold. The RNA was then immobilised to the nylon by UV cross-linking in a UV Stratalinker (Stratagene, La Jolla, CA). The membrane was subjected to overnight prehybridisation conditioning by rotation at 42°C in a hybridisation oven. The probe was then added to the hybridisation tube and allowed to incubate overnight with rotation at 42°C. The membrane was then washed four times (two quick rinses at room temperature and two 45 min rinses at 42°C) in a solution of 2× SSC and 0.1% SDS. The membrane was finally rinsed in 2× SSC, wrapped in transparent wrap, and exposed overnight to a Molecular Dynamics (Sunnyvale, CA) PhosphorImager screen. Quantitation of dot intensities was carried out with a Molecular Dynamics model 400B PhosphorImager using ImageQuant software.

RESULTS

The application of ρ° cells as a model system to investigate mtDNA-drug interactions involved treating both ρ° and ρ^+ cells with Adriamycin and comparing the IC_{50} values for each cell type. Cells were treated with Adriamycin at concentrations varying from 0–30 µM of the drug for varying incubation periods and the IC_{50} determined using the SRB assay. The IC_{50} for ρ^+ cells decreased with time from 640 nM for a 2 day exposure to 20 nM for a 6 day exposure, whereas the IC_{50} for ρ° cells decreased only slightly with time, exhibiting an average of approximately 750 nM (Fig. 1B). After 6 days of exposure to Adriamycin, the IC_{50} for ρ° cells was 45-fold higher than that for ρ^+ cells (Fig. 1A). A similar response was also observed between ρ° and ρ^+ cells with vincristine and taxol, drugs that do not act directly on DNA (data not shown).

One explanation of these results is that the drugs were not accumulating normally in ρ° cells, possibly due to the presence of a multidrug resistance (MDR) phenotype. Adriamycin is a highly fluorescent compound, and its cellular uptake can be readily observed by confocal microscopy [Kawai et al., 1997]. The subcellular distribution of Adriamycin was therefore

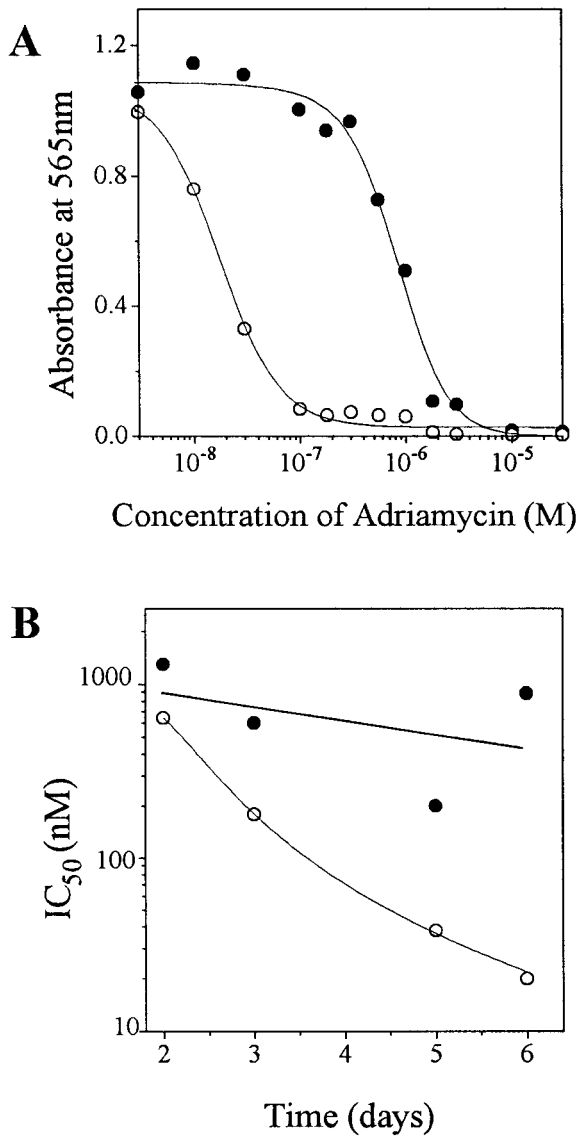

Fig. 1. Time dependence of Adriamycin dose-response. **A:** ρ° (solid circles) and ρ^+ (open circles) rat hepatoma cells were treated with varying concentrations of Adriamycin (3 nM to 30 μM) for 6 days and growth inhibtion measured using the SRB assay. **B:** Dependence of IC_{50} on time of exposure to Adriamycin. Same symbols as in A.

examined in ρ° and ρ^+ cells following exposure to the drug for 4 h. Figure 2A shows the well-known nuclear localisation of Adriamycin in the parental rat hepatoma cells. In contrast, in ρ° cells (Fig. 2C) the total intracellular concentration was dramatically reduced (thirteenfold), and there was essentially no detectable level of drug in the nucleus. However, when both cells were treated with Adriamycin in the presence of the MDR reversal agent SDZ PSC 833, a substrate of P-glycoprotein [Didier and Loor, 1995; Colombo et al., 1996], the extent of uptake of Adriamycin into ρ° cells was similar to that of ρ^+ cells (Fig. 2D,B, respectively). In addition to the well-known and readily observed (Fig. 2) nuclear localisation of Adriamycin, the drug also appeared to localise in mitochondria, as indicated by the cytoplasmic distribution of fluorescence and confirmed by colocalisation of the drug with rhodamine 123 (data not shown), a mitochondrial-specific stain [Johnson et al., 1980].

Since the presence of a MDR phenotype is usually characterised by the upregulation of P-glycoprotein [Licht et al., 1991], mRNA levels for P-glycoprotein were determined by RNA dot blot analysis from total RNA isolated from ρ° and ρ^+ cells. The dot blot (Fig. 3) exhibited a tenfold higher level of P-glycoprotein mRNA in ρ° cells compared to ρ^+ cells, confirming that the rat hepatoma ρ° cells do indeed exhibit the MDR phenotype.

DISCUSSION

The strong dependence of the ID_{50} in ρ^+ cells with increasing time of exposure to Adriamycin and the relative insensitivity of ρ° cells to Adriamycin are consistent with the potential role of Adriamycin as a mtDNA-acting agent, and also with the increasing evidence that mtDNA is the preferred target for a range of other DNA-acting drugs [Murata et al., 1990; Olivero et al., 1995, 1997; Giurgiovich et al., 1997a,b; Wilson et al., 1997]. However, since both vincristine and taxol also exhibited a similar phenomenon and neither of these agents is known to act directly on DNA [Pratt et al., 1994a-c], a likely explanation is that ρ° cells may exhibit the MDR phenotype and thus be inherently resistant to Adriamycin. Evidence for the existence of the MDR phenotype in ρ° cells was therefore sought and was confirmed by two independent observations that the resistance of ρ° cells to Adriamycin was indeed removed by the known MDR-reversal agent SDZ PSC 833 and that there was a tenfold increase in the mRNA level for P-glycoprotein in ρ° cells.

The upregulation of P-glycoprotein in ρ° cells is expected during the process of continual exposure to ethidium bromide used to induce the ρ° state but was also expected to be rapidly diminished following removal of the MDR-inducing agent, as observed with other drugs which induce the MDR phenotype [Licht et al., 1991]. Since the ρ° cells had been passaged some 20–30

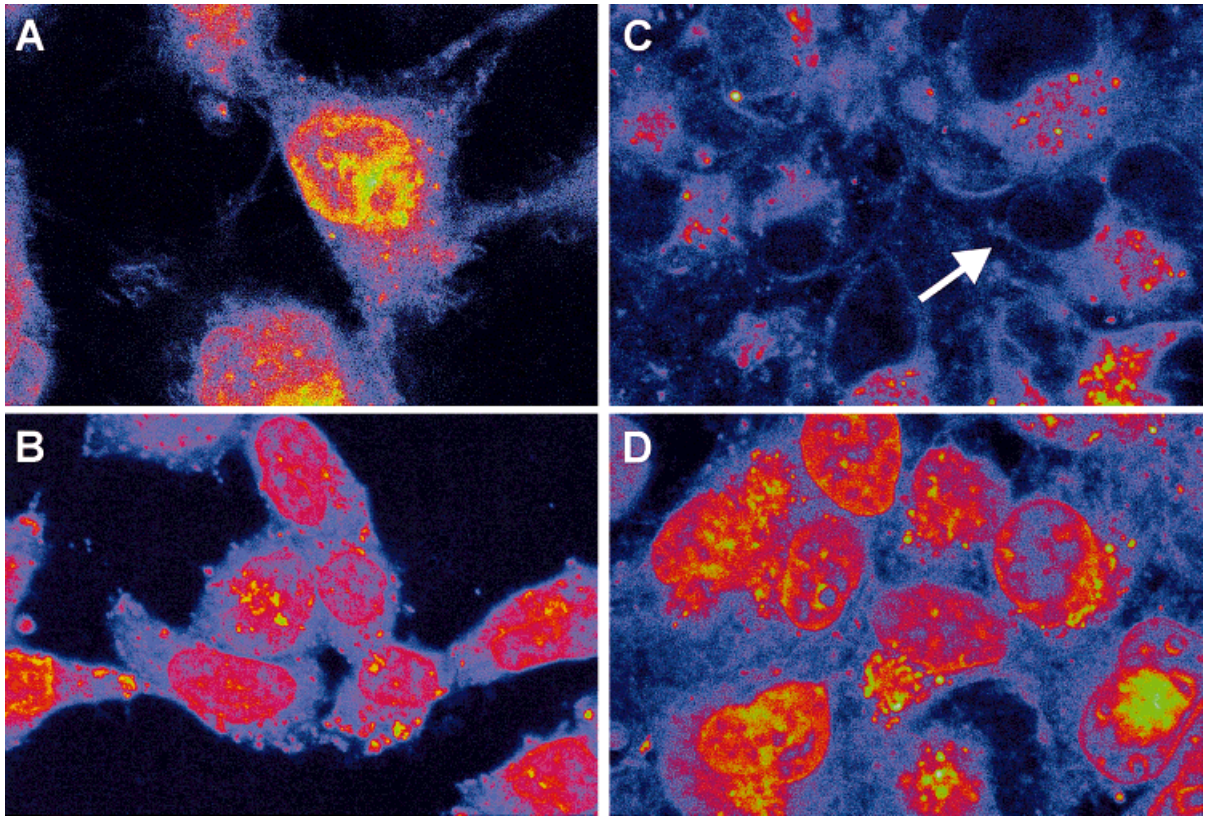

Fig. 2. Confocal microscope images of Adriamycin in rat hepatoma cells. Approximately 5 × l0^6 cells (A–D) were allowed to attach overnight to a two-compartment slide chamber and then exposed to 10 μM Adriamycin for 4 h. **A:** ρ^+ cells. **B:** ρ^+ cells pretreated with the MDR reversal agent, SDZ PSC 833 (1 μM), for 30 min. **C:** ρ° cells. **D:** ρ° cells pretreated with SDZ PSC 833 (1 μM) for 30 min. Increasing concentrations of Adriamycin are represented by the colour scale from black (no drug), to purple, red, orange, and green (highest concentration). The arrow in C indicates the nucleus of a single cell and shows the absence of drug in the nucleus in ρ° cells.

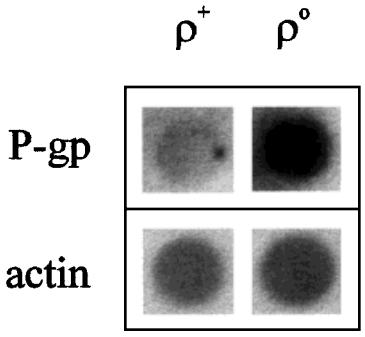

Fig. 3. Northern dot blot analysis for P-glycoprotein. Total cellular RNA was isolated from ρ° and ρ^+ rat hepatoma cells, and 40 μg from each cell line was dotted onto a nylon membrane using a dot blot manifold and probed with ^{32}P-labelled P-glycoprotein (P-gp) and actin probes.

times prior to this study and the upregulation of P-glycoprotein continued to be detected for several more months in cells not exposed to ethidium bromide, this indicates that the elevated level of P-glycoprotein in these cells is extremely long-lived following removal of the initial ρ°-inducing agent. In this context, it is interesting to note that other cellular stresses such as heat shock, X-ray irradiation, and arsenite treatment induce increased *MDR1* mRNA and/or P-glycoprotein levels [Chin et al., 1990; Ciocca et al., 1992; Kioka et al., 1992; Hill et al., 1994; Venetianer et al., 1994], and it is not yet known if the characteristics of the MDR phenotype derived from general cellular stresses are the same as those induced by long-term exposure to DNA intercalating agents.

Recently, a cisplatin-resistant human carcinoma cell line, has been described which has no expression of the *MDR1* gene, and in these cells the resistance to cisplatin was proportional to the level of induction of mitochondrial cpn60 [Shen et al., 1995]. However, since these cells were also shown to be cross-resistant to Adriamycin, this suggests a possible role for mitochondrial cpn60 in the resistance to Adriamycin.

The upregulation of a mitochondrial-specific chaperonin (cpn60) could be reflective of cisplatin inducing an organelle-specific stress response, and Martinus et al. [1996] have indeed demonstrated the existence of an organelle-specific stress response in ρ° cells. The resistance to Adriamycin in ρ° cells shown in this study is therefore likely to be due to a complex mechanism involving the interplay between induction of P-glycoprotein and the induction of cellular stress responses in addition to the other known anthracycline-resistance mechanisms [Nielsen et al., 1996].

There are therefore several important conclusions from this study:

1. ρ° cells exhibit a longer-lived upregulation P-glycoprotein than expected (based on previous decay rates following removal of the resistance-inducing agent).
2. Resistance to Adriamycin may be due to two potentially independent processes, one involving an active efflux process (P-glycoprotein) and another as yet unknown process involving cpn60.
3. ρ° cells are not a suitable model system for the study of drugs known to induce the MDR phenotype or any drug which induces a sufficient cellular stress to upregulate cpn60.

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