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Cell Membrane Transport of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the Liver and Systemic Bioavailability

Maria C. Yang,*,[1] Allan J. McLean,*,† and David G. Le Couteur†,‡

**Canberra Clinical School of the University of Sydney, Canberra Hospital, Garran, ACT, Australia 2065; †John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia 0200; and ‡Centre for Education and Research on Ageing, and ANZAC Research Institute, Concord R. G. Hospital, New South Wales, Australia 2139*

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Modulation of hepatic disposition of MPTP could influence susceptibility to its neurotoxicity. Therefore, we studied hepatocellular transport of MPTP in the perfused rat liver and isolated rat hepatocytes. The perfused liver extensively extracted MPTP. Amiloride and tubocurarine, inhibitors of OCT1, increased MPTP recovery (253 ± 78 and 283 ± 64%, respectively) and reduced PS_{influx} (0.69 ± 0.36 to 0.27 ± 0.11, and 0.97 ± 0.50 to 0.23 ± 0.05 ml/s/g, respectively). P-glycoprotein inhibitor, daunomycin, and Oatp 1 & 2 inhibitor, rifamycin, had no effect. In isolated hepatocytes, amiloride and tubocurarine increased hepatic uptake of MPTP (23 ± 12 and 6 ± 2%, respectively). Daunomycin reduced MPTP uptake by 22 ± 8% and rifamycin had no effect. Only a small proportion of MPTP is taken up into hepatocytes by transporters; however, modulation of these transport mechanisms will influence systemic bioavailability.

Key Words: **MPTP; Parkinson's disease; neurotoxins; pesticides; hepatocytes; membrane transporters; perfused rat liver; multiple indicator dilution.**

PD is a common neurodegenerative disorder characterised pathologically by loss of dopaminergic neurones and formation of Lewy bodies within the pars compacta of the substantia nigra (1, 2). PD may have a neurotoxic pathogenesis. Evidence for this includes the observation that PD is common among people with a history of exposure to pesticides (3–5) and two neurotoxins, MPTP and rotenone, have been shown to induce experimental parkinsonian syndromes (6–8). Both MPTP and rotenone interact *in vitro* with α-synuclein, a protein found in Lewy bodies and associated mechanistically with several forms of inherited parkinsonian syndromes (8–10).

MPTP is now an established experimental model for PD. MPTP is a cyclic tertiary allylamine that has been shown to produce a parkinsonian syndrome and dopaminergic cell death in humans (11), nonhuman primates (12) and various rodents (13). The mechanism of action and disposition of MPTP has been extensively investigated (14–16). MPTP is detoxified, presumably primarily in the liver, by cytochrome P450 (CYP) (17, 18) and flavin-containing monooxygenase (FMO) enzymes (19, 20). MPTP that escapes systemic metabolism can cross the blood–brain barrier because it is lipophilic (21). MPTP is a pro-toxin that is activated to MPP^+ by monoamine oxidase B (MAOB) (22, 23). MPP^+ is selectively transported into dopaminergic cells by the dopamine transporter, then binds complex I of the mitochondrial electron transfer chain, producing cell death as a result of ATP depletion and oxidative stress (24).

The effect of the blood brain barrier is critical because MPP^+, which is unable to penetrate the blood–brain barrier, is only neurotoxic when administered via an intracerebral route, whereas MPTP causes neurotoxicity when administered systemically. Variation in the systemic disposition of MPTP is thought to influence susceptibility to MPTP-induced neurotoxicity by affecting the amount of MPTP that is delivered to the blood-brain barrier. Modulation of hepatic metabolism of MPTP by inhibitors of CYP and FMO alters susceptibility to MPTP neurotoxicity (25, 26), and, furthermore genetic polymorphism in CYP 2D6 (27) and other xenobiotic metabolising enzymes influence risk of PD in humans (28, 29). The liver extensively extracts MPTP from the portal vein therefore small changes in hepatic extraction of PTP will have profound effects on systemic exposure (30) and, hence, neurotoxicity.

[1] To whom correspondence and reprint requests should be addressed at Department of Geriatric Medicine, Canberra Hospital, Yamba Drive, Garran, ACT, Australia 2605. Fax: +612 6244 4036. E-mail: Maria.Yang@anu.edu.au.

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Although the hepatic metabolism of MPTP has been investigated there are few reports on the hepatocellular transport of MPTP. Because of the possibility that modulators of hepatic cellular transport of MPTP might also influence susceptibility to MPTP-induced neurotoxicity we studied transport mechanisms for MPTP in the intact perfused rat liver and isolated hepatocytes.

MATERIALS AND METHODS

Animals. Male Wistar rats (2–3 months old [200–400 g], John Curtin School of Medical Research, Canberra, Australia) were maintained on standard rat food pellets, water *ad libitum*. The study was approved by the Australian National University Animal Experimentation Ethics Committee.

Chemicals. Methyl-4-phenyl-1,2,3,6-tetrahydropyridine,1-[methyl-^{3}H] was purchased from ARC (Missouri) and [U-^{14}C]-sucrose and daunomycin from ICN (California). Amiloride, d-tubocurarine chloride, rifamycin and collagenase were obtained from Sigma (Missouri).

Disposition of ^{3}H-MPTP in the perfused rat liver. The liver perfusion and MID technique were used to determine the disposition of MPTP in the perfused rat liver, as we have described previously (30). Briefly, the livers were perfused *in situ* via the portal vein with Krebs–Henseleit buffer containing 1% bovine serum albumin (BSA) in a single pass mode. The perfusate flow rate was maintained at 19–21 ml/min and the injectate (50 μl), containing ^{3}H-MPTP and ^{14}C-sucrose (0.5 μCi), was administered as a bolus through the portal vein catheter. ^{14}C-sucrose was the extracellular marker. Outflow samples from the thoracic inferior vena cava were collected for 60 s after the injection and analysed with a scintillation counter. Additional experiments were performed in the same livers after ten minutes pretreatment with either 1 mM amiloride, 200 μM tubocurarine, 100 μM daunomycin or 10 μM rifamycin. Amiloride and tubocurarine are inhibitors of OCT1. Daunomycin is an inhibitor of a multi-drug resistance gene product (MDR) also known as P-glycoprotein. Rifamycin is an inhibitor of Oatp 1 & 2.

Analysis of multiple indicator-dilution experiments. The hepatic outflow concentrations were expressed as the fraction of the injected dose per ml. The recovery of the ^{3}H-MPTP in the effluent was determined from the area under the outflow curve (AUC). The rate constants for the cellular influx, efflux and sequestration (k_1, k_2, and k_3, respectively), and the permeability-surface area products for the hepatocellular influx (PS_{influx}) and efflux (PS_{efflux}) were determined using the distributed models developed by Goresky (31) as we have described previously (30). The volume of distribution for ^{3}H-MPTP in the liver was calculated from that of sucrose, k_1 and k_2.

Uptake of ^{3}H-MPTP into rat hepatocytes. Hepatocytes were isolated according to a procedure described previously (32). Livers were perfused for 2 min with calcium-free Krebs–Henseleit buffer saturated with 95% O_2/5% CO_2 at a flow rate of 40 ml/min. Then, perfusion was continued for 8 min with collagenase (100 mg in 150 ml buffer). The liver was removed and digested at 37°C for 5 min in collagenase buffer containing 1 mM calcium. The cells were sieved and washed three times at 4°C. Cell viability was determined using trypan blue (0.16%). Preparations containing more than 90% viable cells were used.

Cells (2×10^6 cells/ml) in triplicate were pre-incubated for 5 min at 37°C in Krebs–Henseleit buffer containing 1% BSA and equilibrated with 95% O_2/5% CO_2. Cells were incubated for five more min with either 1 mM amiloride, 200 μM tubocurarine, 100 μM daunomycin or 10 μM rifamycin. Then 0.3 μCi ^{3}H-MPTP was added to the cell suspension and incubation continued for another 5 min. The cells were washed three times and lysed in perchloric acid (0.2 M). Radioactivity was counted using a liquid scintillation counter (Packard Instruments, U.S.A.). The effect of each agent on MPTP uptake was calculated as a percentage of the radioactivity in each sample compared to that in the control samples. Experiments were replicated four times.

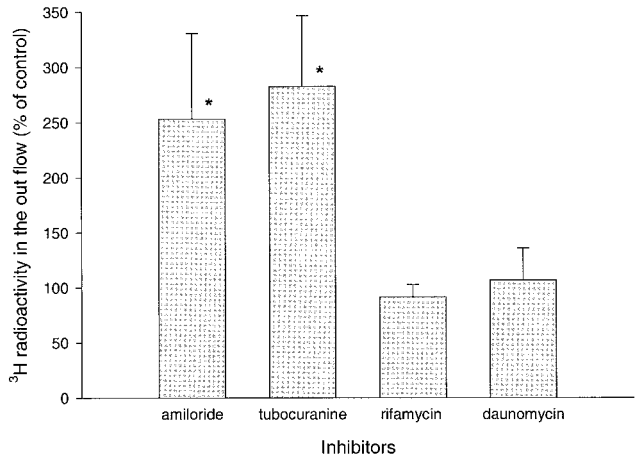

FIG. 1. The effect of hepatocyte membrane transporter inhibitors on the recovery of MPTP in the perfused rat liver. Results are shown as percentage of the control (100%) value (mean ± SD). *Significantly different from control ($P \leq 0.05$).

Statistics. All data are presented as means ± SD. The paired and unpaired Student *t* test were used to compare results and considered significant when $P \leq 0.05$.

RESULTS

Disposition of ^{3}H-MPTP in the Perfused Rat Liver

Figure 1 shows the effect of cell membrane transporter inhibitors on the recovery of ^{3}H-MPTP in the effluent of perfused rat livers. The OCT1 inhibitors, amiloride and tubocurarine, increased the recovery of ^{3}H-MPTP in the outflow by 253 ± 78% ($P = 0.01$) and 283 ± 64% ($P = 0.004$), respectively. The P-glycoprotein inhibitor, daunomycin, and the Oatp1 & 2 inhibitor, rifamycin, did not have any significant effects on the recovery of ^{3}H-MPTP (92 ± 11 and 107 ± 29%, respectively).

Figure 2 shows representative outflow concentration-time profiles before and after the addition of amiloride to the perfusate. The increase in the size of the ^{3}H-MPTP outflow curve after the addition of amiloride is clearly evident.

The values for recoveries and volumes of distribution are shown in Table 1. The recovery of the extracellular marker sucrose was approximately 100% and not influenced by the addition of membrane transport inhibitors. The recovery of ^{3}H-MPTP in control experiments was 2.5–4.1%, indicating that the hepatic extraction of MPTP is extremely efficient. The volume of distribution of MPTP was approximately 3 ml/g of liver and this was reduced significantly after treatment with amiloride (3.84 ml/g in control versus 1.70 ml/g liver

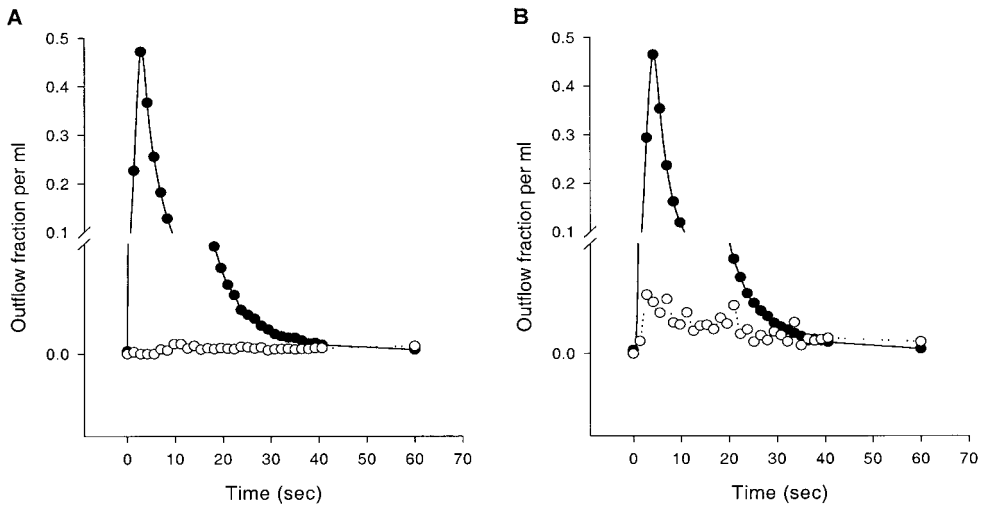

FIG. 2. The effect of amiloride on the outflow curves of MPTP in the perfused rat liver. The injectate consisted of ^{3}H-MPTP (open circles) and ^{14}C-sucrose (closed circles). The experiments were performed in the absence (A) and presence (B) of amiloride in the same liver.

after addition of amiloride, $P = 0.05$) but not the other agents.

Table 2 shows the values for k_1, k_2, k_3, PS_{influx} and PS_{efflux} of ^{3}H-MPTP in the perfused liver. The OCT1 inhibitors, amiloride and tubocurarine reduced the value of k_1 and PS_{influx} substantially, indicating that these agents increased the recovery of MPTP by inhibiting influx. PS_{efflux} was reduced by tubocurarine and although this was not statistically significant, it may indicate that MPTP efflux also occurs via OCT1. Neither agent significantly influenced k_3. Daunomycin and rifamycin had no significant effects on k_1, k_2, k_3, PS_{influx} and PS_{efflux} of ^{3}H-MPTP.

Disposition of ^{3}H-MPTP in Isolated Rat Hepatocytes

Figure 3 shows the effects of the transporter inhibitors on the uptake of ^{3}H-MPTP into the hepatocytes. The results are quite different from those seen in the perfused liver. Amiloride and tubocurarine increased hepatic uptake of ^{3}H-MPTP (123 ± 12%, $P = 0.08$ and 106 ± 2% of control values, respectively, $P = 0.002$). Daunomycin reduced hepatocyte uptake to 78 ± 8% of control values ($P = 0.001$) and rifamycin did not have any effect. Overall, in the control experiments, 78% of the radioactivity was taken up by isolated hepatocytes.

DISCUSSION

The transport of xenobiotics across the plasma membrane of hepatocytes is an important determinant of hepatic elimination (33, 34). Uncharged lipophilic molecules are transported largely by passive diffusion (33) and many transporter mechanisms are present for other classes of xenobiotics (35, 36). Here, we investigated the hepatic transport of MPTP because it is implicated in the pathogenesis of Parkinson's disease,

TABLE 1

Fractional Recoveries of Sucrose and MPTP, and Volume of Distribution of MPTP in the Perfused Rat Liver before and after Treatment with Membrane Transport Inhibitors

Inhibitors		*N*	Recovery of sucrose (%)	Recovery of MPTP (%)	Volume of distribution of MPTP (ml/g liver)
Amiloride	Control	6	98 ± 4	3.7 ± 3.1	3.84 ± 1.63
	Test	5	96 ± 4	9.2 ± 2.8* ($P = 0.01$)	1.70 ± 1.47* ($P = 0.05$)
Tubocurarine	Control	4	100 ± 3	2.5 ± 0.9	2.15 ± 0.24
	Test	3	104 ± 2	7.2 ± 1.6* ($P = 0.004$)	2.25 ± 0.57
Daunomycin	Control	3	98 ± 2	4.2 ± 0.2	2.73 ± 0.23
	Test	3	98 ± 3	4.5 ± 1.2	2.59 ± 0.83
Rifamycin	Control	4	100 ± 3	4.1 ± 0.3	3.09 ± 0.24
	Test	4	99 ± 1	3.7 ± 0.5	2.25 ± 0.62

* Statistically different from controls $P \leq 0.05$.

TABLE 2

The Effects of Membrane Transport Inhibitors on k_1, k_2, k_3, PS_{influx}, and PS_{efflux} in the Perfused Rat Liver

Inhibitors		k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	PS_{influx} (ml/s/g liver)	PS_{efflux} (ml/s/g liver)
Amiloride	Control	4.3 ± 2.5	0.3 ± 0.2	0.2 ± 0.3	0.69 ± 0.36	0.22 ± 0.21
	Test	1.4 ± 0.6* ($P = 0.03$)	0.4 ± 0.3	0.2 ± 0.2	0.27 ± 0.11* ($P = 0.04$)	0.29 ± 0.21
Tubocurarine	Control	6.2 ± 3.8	0.5 ± 0.3	0.1 ± 0.05	0.97 ± 0.50	0.42 ± 0.23
	Test	1.4 ± 0.4 ($P = 0.09$)	0.1 ± 0.04 ($P = 0.06$)	0.1 ± 0.03	0.23 ± 0.05* ($P = 0.05$)	0.10 ± 0.04 ($P = 0.07$)
Daunomycin	Control	2.4 ± 0.6	0.2 ± 0.1	0.1 ± 0.02	0.55 ± 0.21	0.17 ± 0.05
	Test	2.1 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	0.45 ± 0.05	0.16 ± 0.07
Rifamycin	Control	1.7 ± 0.6	0.2 ± 0.1	0.2 ± 0.1	0.48 ± 0.22	0.12 ± 0.06
	Test	2.0 ± 0.6	0.3 ± 0.2	0.2 ± 0.1	0.53 ± 0.19	0.22 ± 0.11

* Statistically different from controls $P \leq 0.05$.

and because of the possibility that modulation of hepatic transporters might influence susceptibility to neurotoxins by altering bioavailability and systemic exposure.

MPTP was extensively taken up by both the perfused liver and isolated hepatocytes. The extraction of MPTP by the perfused liver was greater than 95% and for isolated hepatocytes was nearly 80%. As we have concluded previously, it is likely that MPTP, which is a lipophilic molecule, is mostly taken up by the liver via simple diffusion (30). Even so, we found that inhibitors of hepatocyte membrane transporters did influence uptake to a significant extent, indicating that other mechanisms apart from diffusion are present. The difference in the uptake by isolated hepatocytes and the perfused liver might represent the influence of transport by non-parenchymal cells, which represent about 20% of liver volume (37).

We found that OCT1 is involved in MPTP transport. There are two OCTs in the sinusoidal membrane of the hepatocyte. Type 1 OCT is involved in the uptake of relatively small monovalent organic cations such as MPP^+ (38) and procainamide (39), while the type 2 OCT accepts organic cations with bulky ring structures, such as vecuronium (40). The inhibitors of OCT1, amiloride and tubocurarine, increased the recovery of MPTP from the perfused liver from about 3–4% to about 7–9%. Furthermore, this was associated with a substantial reduction in the values for PS_{influx}, confirming that this effect was mediated by inhibition of the inward transport of MPTP. Overall, the results suggest that approximately 5% of MPTP uptake by the intake liver occurs via OCT1. Although this is a small fraction of the total uptake, inhibition of OCT1 with amiloride and tubocurarine increased the recovery by 250–300%, which will have a profound effect on systemic exposure. By contrast, amiloride and tubocurarine were associated with increased accumulation of MPTP in isolated hepatocytes.

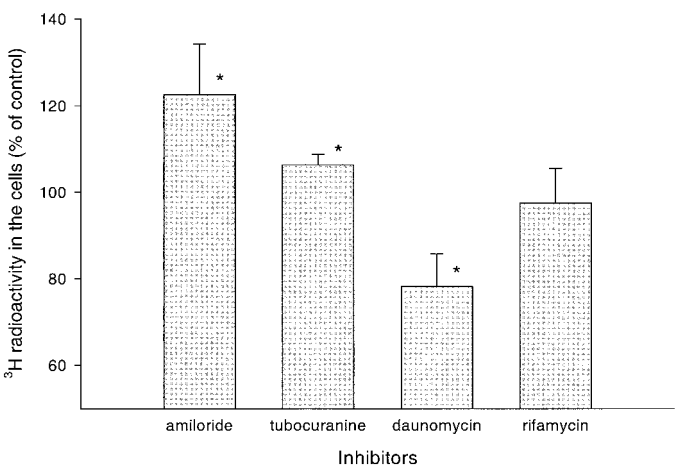

FIG. 3. The effect of cell membrane transporter inhibitors on the uptake of MPTP in isolated hepatocytes. Results are shown as percentage of the control (100%) value (mean ± SD). *Significantly different from control ($P \leq 0.05$).

The observation that OCT1 mediates MPTP influx in the intact liver and efflux in isolated hepatocytes is apparently paradoxical. It is possible that in the isolated hepatocytes, the duration of the experiment is long enough for MPTP to be transformed by hepatic MAOB to MPP^+, which is effluxed by OCT1. However, the transformation of MPTP to MPP^+ by hepatocytes appears to occur over an even longer time course than that which we used, and there is thought to be little leakage of MPP^+ from hepatocytes (20). It is also possible that the effects of P-glycoprotein on MPTP transport, which is only seen in isolated hepatocytes, influences the action of OCT1. Finally, it is also possible that there is transport of MPTP via OCT1 by non-parenchymal cells. In renal brush border membranes, MPTP undergoes bidirectional transport via OCT1 (41). In isolated hepatocytes, it has been shown MPP^+ undergoes substantial uptake via OCT1 (38). Our results with the perfused rat liver and isolated hepatocytes show that OCT1 mediates the influx, and possi-

bly bidirectional transport, of a small fraction of total MPTP uptake into hepatocytes.

We found that MPTP transport into isolated hepatocytes is reduced by daunomycin, an inhibitor of P-glycoprotein. P-glycoprotein or MDR is a member of a super family of ATP-dependent canalicular membrane transporters (42, 43). P-glycoprotein in the liver is found on the canalicular surface of the hepatocytes, and is involved with the transport of various substrates (e.g. organic cations (44), glutathione conjugates (45) and glucuronides (46)) from the hepatocyte into the bile. Daunomycin, an inhibitor of p-glycoprotein, did not have any effect on MPTP recovery from the perfused rat liver. This may be because the canalicular membrane is not exposed to P-glycoprotein inhibitors delivered by the portal vein in perfused liver experiments. In isolated hepatocytes, P-glycoprotein may be exposed to inhibitors. The effect of daunomycin in isolated hepatocytes suggests that P-glycoprotein is involved in the unidirectional transport of MPTP into hepatocytes. Again, this is an apparently paradoxical result because P-glycoprotein is generally considered to be an efflux pump. However, it has been reported that MPP^+ is taken up into isolated hepatocytes by P-glycoprotein, with a reduction in the accumulation of MPP^+ to 1% of control values by daunomycin (38). Daunomycin did not have any effect on MPP^+ transport in the perfused liver (38). Alternatively, daunomycin may have actions on other transport mechanisms.

Finally, we found that MPTP transport into hepatocytes is not mediated by Oatp. Oatp1 is a sodium-independent multispecific transporter that mediates hepatocellular uptake of bromosulphophthalein (47) and bile salts (48). Oatp2 is a close homologue of Oatp1 and also transports bile salts and steroid conjugates with partially selective substrate specificities (49). Both Oatp1 & 2 are localised to the basolateral membranes of hepatocytes (49, 50). Rifamycin, an inhibitor of Oatp had no effect on the uptake of MPTP either in the perfused rat liver or isolated hepatocytes and did not influence the parameters for the influx or efflux of MPTP in the perfused liver.

In contrast to MPTP transport, the transport of MPP^+, the neurotoxic metabolite of MPTP, has been well studied (38, 41). MPP^+ is a small organic cation and carrier-mediated active transport systems are necessary for its penetration into cells. OCT1 and P-glycoprotein are involved in MPP^+ transport into hepatocytes (38). MPP^+ is also taken up into canalicular rat liver plasma membrane vesicles by an organic cation/H+ exchanger (51). However, from the point of view of the pathogenesis of Parkinson's disease, the study of MPTP is more important given that MPP^+ is unable to cross the blood brain barrier and variability in systemic exposure to MPP^+ would not be expected to influence neurotoxicity in the substantia nigra.

In summary, MPTP is extracted extensively by the liver. Most uptake into hepatocytes appears to be mediated by simple diffusion; however, a small but potentially significant proportion of uptake is via OCT1 and, possibly in isolated hepatocytes, via P-glycoprotein. Oatp is not involved in MPTP transport, and OCT1 may be a bidirectional transporter involved with efflux of MPTP in isolated hepatocytes. Although only a small fraction of MPTP uptake is mediated by transporters, with extensive hepatic extraction even minor modulation of MPTP transport will have dramatic effects on systemic exposure to MPTP, hence susceptibility to neurotoxicity.

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