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ORIGINAL ARTICLE

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Effects of amifostine on perfused isolated rat heart and on acute doxorubicin-induced cardiotoxicity

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Abstract *Purpose*: To determine the effects of amifostine on an isolated perfused rat-heart model and its protective activity with regard to cardiotoxic doxorubicin perfusion. *Methods*: Langendorff constant-pressure isolated rat-heart preparations were used to analyze the effects of the drugs during a 40-min period of perfusion after a 20-min stabilization interval. The first study was conducted with amifostine alone (controls and 10^{-6}, 10^{-5}, and 10^{-4} *M* amifostine; $n = 6$ in each group). The second study was conducted with amifostine and doxorubicin (controls, 2.5×10^{-5} *M* doxorubicin, 2.5×10^{-5} *M* doxorubicin and 10^{-5} *M* amifostine, and 2.5×10^{-5} *M* doxorubicin and 10^{-4} *M* amifostine; $n = 4$ in each group). *Results*: Amifostine had no significant effect on hemodynamic parameters at 10^{-6}, 10^{-5}, and 10^{-4} *M* concentrations. However, amifostine increased the coronary flow expressed as a percentage ± SEM of the baseline flow as follows: 82 ± 4% for controls, 95 ± 6% for 10^{-6} *M* amifostine, ($P = 0.13$), 111 ± 4% for 10^{-5} *M* amifostine ($P < 0.01$), and 104 ± 3% for 10^{-6} *M* amifostine ($P < 0.01$). When we commenced an amifostine perfusion 20 min in advance of and then during a 40-min perfusion with doxorubicin, at a cardiotoxic concentration of 2.5×10^{-5} *M* the left ventricular pressures (LVDP, expressed as percentages ± SEM of the baseline LVDP before doxorubicin) were 55 ± 3% for the doxorubicin controls, 68 ± 2% for doxorubicin with 10^{-5} *M* amifostine ($P = 0.05$), and 80 ± 3% for doxorubicin with 10^{-4} *M* amifostine ($P < 0.01$). Whether this protective effect might be related to the known free-radical-scavenging activity of amifostine remains to be determined. *Conclusion*: On a Langendorff-type model of rat heart, 10^{-5} and 10^{-4} *M* amifostine alone induced a coronary dilation and, when associated with a cardiotoxic concentration of 2.5×10^{-5} *M* doxorubicin, 10^{-5} and 10^{-4} *M* amifostine displayed a cardioprotective effect.

Key words Amifostine · Doxorubicin · Isolated rat heart · Cardiotoxicity · Cardioprotection

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Introduction

Amifostine (WR-2721) is a cytoprotective agent that selectively protects normal tissue from the toxicity of chemotherapic agents such as cisplatin or cyclophosphamide [11, 12] and from that of radiation therapy [16, 22]. In a clinical setting, amifostine's cytoprotective activity has been used during cisplatin and cyclophosphamide chemotherapy, reducing hematologic and renal toxicity [12]. Alkaline phosphatase in the cellular membrane of endothelial cells of small vessels induces the dephosphorylation of amifostine into WR-1065, an aminothiol compound that crosses the cellular membrane [23] and is probably responsible for normal tissue protection [22]. The poor vascularization of solid tumors, together with their decreased alkaline phosphatase activity and lower pH, may explain the selective protection of nontumoral tissues [5]. Studies of intracellular glutathione and creatine-phosphokinase activities have suggested a cardioprotective effect for amifostine with regard to doxorubicin's toxicity to cultured rat-heart myocytes [7] and in tumor-bearing BALB/c mice [3]. Amifostine has been shown to scavenge hydroxyl radicals in a model of cultured rat-heart myocytes following exposure to doxorubicin [7]. Amifostine also demonstrates cardioprotective activity in the postischemic isolated rat heart via its radical-scavenging activity, inducing a better recovery of hemodynamic function together with higher creatine phosphate levels [18]. However, lacking are studies on the effects of amifostine on heart models exposed or not exposed to anthracy-

clines. To appreciate the possible cardiac protection from anthracycline toxicity provided by amifostine we carried out a study to characterize amifostine's effects on the isolated rat heart when applied singly and together with doxorubicin. For this purpose the isovolumic perfused rat heart according to Langendorff was used together with simultaneous recording of both the contractile performance and the oxygen consumption of the heart [1].

Materials and methods

Reagents

Amifostine was a gift from U.S. Bioscience (USA), and doxorubicin was a gift from Pharmacia (France). Both products were first dissolved in sterile water and then added to the Krebs-Henseleit solution before oxygenation and warming.

Isolated heart preparation

Male Wistar rats aged 2 months, weighing 280–320 g, and fed ad libitum were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneal) and heparinized with a 500-IU intravenous injection. The hearts were promptly excised, placed in oxygenated Krebs-Henseleit buffer at 37 °C, and perfused by aortic cannulation according to the non-recirculating Langendorff technique. We used the balloon method for recording of isovolumetric pressure in the isolated perfused heart [8]. In brief, after the start of the perfusion a latex balloon was inserted into the left ventricle through the left atrium and connected to a pressure transducer (Spectramed model P10EZ transducer, Gould 8000S recorder; Gould Electronics, Ballainvilliers, France). The balloon was filled with distilled water such that the initial diastolic pressure was set at 8 ± 2 mmHg. The perfusion pressure was kept constant at 9.8 kPa, and the hearts were paced at a constant rate by a coaxial electrode placed in contact with the heart, the tissue being stimulated between the inner platinum wire and the outer cylinder of the electrode (ref. cat 13; Hugo Sachs Elektronik KG, March, Germany).

Experimental protocol

All hearts were allowed to equilibrate for 20 min on the first perfusion column with standard Krebs-Henseleit solution (118 m*M* NaCl, 4.7 m*M* KCl, 1.25 m*M* $MgSO_4$, 1.2 m*M* $CaCl_2$, 1.2 m*M* KH_2PO_4, 22.6 m*M* $NaHCO_3$, 11.1 m*M* glucose, equilibrated at a pH value of 7.4). The perfusate was continuously bubbled with 95% O_2 and 5% CO_2 and was kept at 37 °C (Thermomix 1419, Braun). The beat rate was set to 300/min by pacing; if the spontaneous rhythm was faster, the rate was set to the lowest heart rate allowing constant pacing. Measurements recorded after the 20-min stabilization period (t20) were considered to be baseline values. In the first group, from 20 min to 60 min, hearts were exposed to amifostine (10^{-6}, 10^{-5}, or 10^{-4} *M*; $n=6$ for each concentration) diluted in Krebs-Henseleit perfusate or to the vehicle (controls, $n=6$) by switching to the second perfusion column. In the second group, hearts were exposed from 20 min to 60 min to doxorubicin (2.5×10^{-5} *M*) alone (doxorubicin controls, $n=4$) or together with 10^{-5} or 10^{-4} *M* amifostine in the Krebs-Henseleit solution ($n=4$ for each concentration) from the second perfusion column. In this group, stabilization was performed with the same amifostine dose regimen, whereas doxorubicin controls were perfused with Krebs-Henseleit solution only. The control group ($n=4$) was perfused with Krebs-Henseleit solution during stabilization (first column) and thereafter (second column).

Measurements

Contractile parameters

The contractile parameters were measured every 10 min from 20 min to 60 min. We measured the systolic and diastolic left ventricular pressures; their difference defined the left ventricular developed pressure (LVDP). The positive and negative differentiated pressures were simultaneously measured by the same differentiator (Gould 8000S; Gould Electronics, Ballainvilliers, France). The maximal value recorded for the contraction rate (+ dP/dt max) was used as an inotropism index, and the minimal value (−dP/dt max) served as a relaxation index. The data were expressed as percentages of the baseline values measured at t20 at the end of the stabilization period.

Oxygen consumption and coronary flow

The mean coronary flow was measured every 10 min from 20 min to 60 min; samples of coronary effluent were collected from the right ventricle, and the mean coronary flow was measured by collection of the coronary effluent for 1 min. At 20, 30, 40, and 60 min the oxygen partial pressure was measured from the perfusate and from coronary effluent samples with a Clark-type electrode connected to an oxymeter (Diamond's General's Chemical Microsensor), which allowed us to assess the oxygen extraction rate and the oxygen consumption rate expressed in micromoles per minute per gram of heart dry weight. These values were also expressed as percentages of the baseline values measured at t20.

Extratissual creatine kinase determination

Creatine kinase (CK) release was measured after the stabilization period (20 min) and after 30, 40, and 60 min in coronary effluent samples; the area under the curve was then calculated and expressed in international units (μIU) per gram of heart dry weight. Measurements were based on a fully enzymatic method using a commercially available reagent pack (Boehringer Mannheim ref. 1 087 533).

Statistical analysis

All data are presented as mean percentages of baseline value ± SEM; the overall statistical significance for differences across the groups was tested by one- or two-factor (amifostine and doxorubicin dose regimen) analysis of variance for repeated measures. If the difference was significant ($P < 0.05$), each group was then compared with the control group using Student's *t*-test.

Results

Base line values appear in Table 1.

Amifostine's effects

We found no significant hemodynamic effect for amifostine. LVDP, +dP/dt max, and −dP/dt max did not differ from control values when amifostine was perfused (Fig. 1). A significant increase in coronary flow was found at the two highest doses of amifostine (controls 82 ± 4%; 10^{-6} *M* amifostine 95 ± 3%, $P=0.12$; 10^{-5} *M* amifostine 111 ± 4%, $P < 0.01$; 10^{-4} *M* amifostine 104 ± 3%, $P=0.01$) together with a decrease in oxygen extraction (controls 100 ± 2%; 10^{-6} *M* amifostine 97 ± 2%, $P=0.42$; 10^{-5} *M* amifostine 81 ± 2%, $P < 0.01$; 10^{-4} *M* amifostine 92 ± 4%,

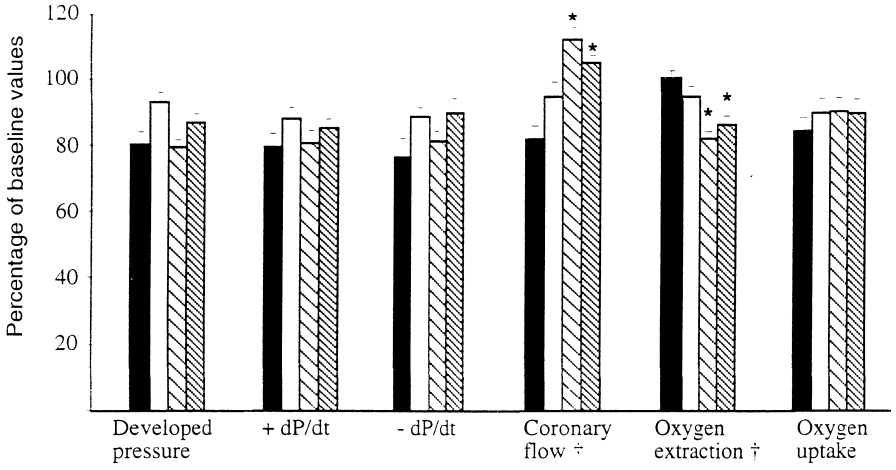

Fig. 1 Acute effects of amifostine on isolated rat heart after a 40-min period of perfusion. † $P < 0.05$ for one factor (amifostine), ANOVA; *$P < 0.05$ as compared with the control group (■ Controls, □ 10^{-6} *M* amifostine, ▨ 10^{-5} *M* amifostine, ▧ 10^{-4} *M* amifostine)

$P < 0.01$), whereas the ratio of the heart rate-LVDP product to the oxygen uptake was similar to that found for the controls.

Doxorubicin's effects

Preliminary experiments had shown no hemodynamic effect for 10^{-5} *M* doxorubicin during a 40-min period of perfusion. With 2.5×10^{-5} *M* doxorubicin we observed a significant decrease in inotropism (LVDP, +dP/dt, −dP/dt) after 20 min of doxorubicin perfusion (LVDP at 20 min: controls 91% ± 5%, doxorubicin 77% ± 2%, $P < 0.01$) and thereafter with no significant change in coronary flow or oxygen uptake being noted (Fig. 2). No significant change was found in CK release (control 3,994 ± 889 μIU/g; doxorubicin 3,071 ± 751 μIU/g, $P = 0.11$).

Amifostine and doxorubicin

Contractile function

When amifostine was perfused before and during the perfusion of 2.5 10^{-5}*M* doxorubicin, the doxorubicin-induced decrease in inotropism was diminished (Fig. 2). The improvement was significant for 10^{-5} and 10^{-4} *M* amifostine and appeared to be dose-related. At the

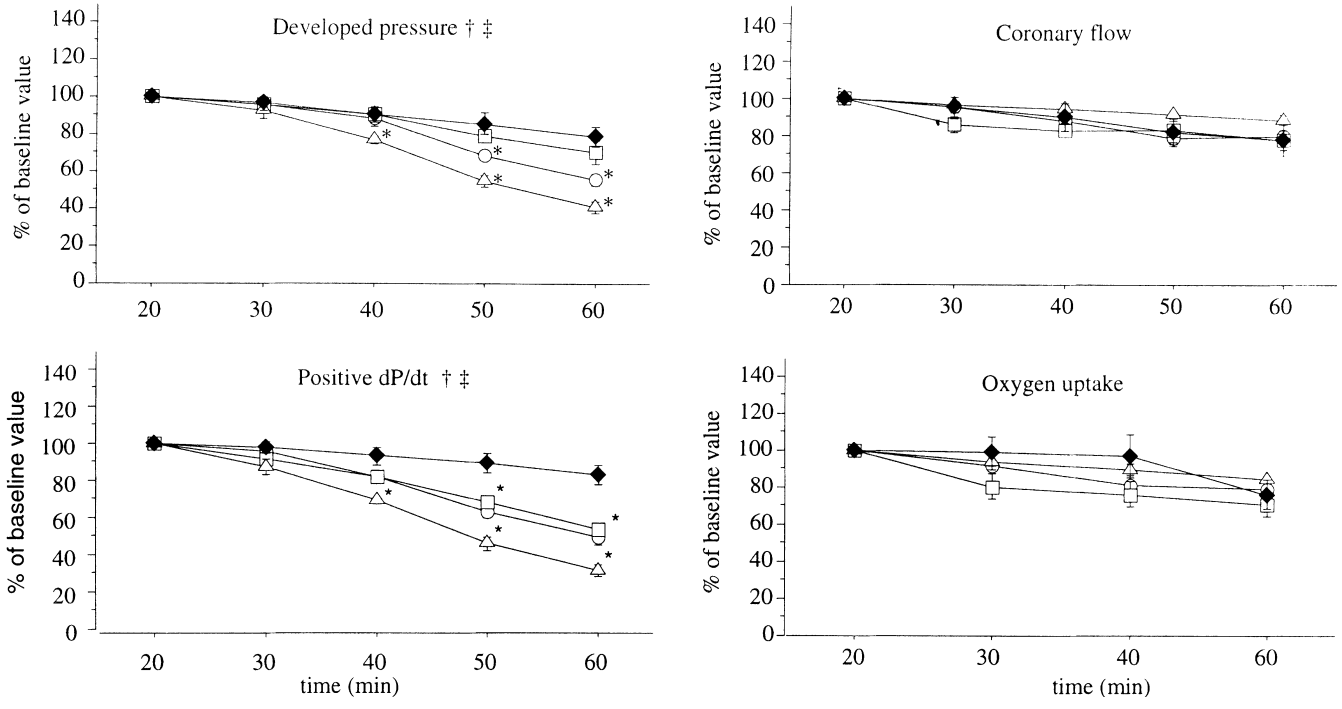

Fig. 2 Acute effects of doxorubicin with and without amifostine on isolated rat heart. †$P < 0.05$ for amifostine; ‡$P < 0.05$ for doxorubicin in two factors, ANOVA; *$P < 0.05$ as compared with the control group at the same time (-◆- Controls, –△– 2.5×10^{-5} *M* doxorubicin, –○– 10^{-5} *M* amifostine and 2.5×10^{-5} *M* doxorubicin, –□– 10^{-4} *M* amifostine and 2.5×10^{-5} *M* doxorubicin)

Table 1 Baseline values obtained in the first (amifostine effects alone) and second (amifostine and 2.5×10^{-5} *M* doxorubicin) experiments (± SD). Baseline values were set after a 20-min period of perfusion with Krebs-Henseleit solution alone. There was no significant difference between controls and the other groups

	Group	Developed pressure (mmHg)	+dPdt (mmHg min^{-1})	−dPdt (mmHg min^{-1})	Coronary flow (ml min^{-1})	Oxygen extraction (%)	Oxygen uptake $\mu M\ min^{-1}\ g^{-1}$
Experiment I	Controls	109 ± 21.1	2,733 ± 294	2,241 ± 405	13.7 ± 3.3	72 ± 9	9,599 ± 1,810
	10^{-6} *M* amifostine	94.6 ± 23.2	2,335 ± 478	1,814 ± 488	11.9 ± 3.8	75 ± 8	9,201 ± 2,939
	10^{-5} *M* amifostine	95.5 ± 19.4	2,716 ± 270	2,375 ± 264	12.8 ± 3.1	73 ± 8	8,891 ± 1,835
	10^{-4} *M* amifostine	99.3 ± 21.4	2,471 ± 303	2,042 ± 251	13.3 ± 4.2	75 ± 6	10,082 ± 3,215
Experiment II	Controls	95.2 ± 17.4	2,225 ± 693	1,833 ± 665	13.2 ± 5.9	71 ± 9	8,924 ± 2,574
	Doxorubicin 2.5×10^{-5} *M*	101.3 ± 26	2,542 ± 531	1,962 ± 524	12.2 ± 1.4	77 ± 5	9,299 ± 1,551
	Doxorubicin and 10^{-5} *M* amifostine	96.4 ± 19.5	2,336 ± 502	1,829 ± 481	13.5 ± 1.3	71 ± 7	9,646 ± 1,758
	Doxorubicin and 10^{-4} *M* amifostine	87.8 ± 24.5	2,554 ± 531	1,942 ± 571	14.4 ± 2.7	69 ± 5	10,106 ± 2,416

highest dose (10^{-4} *M* amifostine), LVDP and −dP/dt did not differ significantly from the control values (LVDP at 40 min: controls 86 ± 6%; doxorubicin 55 ±3%, $P < 0.01$; with 10^{-5} *M* amifostine 68 ± 2%, $P < 0.01$; with 10^{-4} *M* amifostine 80 ± 3%, $P = 0.23$). In contrast, the improvement was highly significant in a comparison with the group treated with doxorubicin alone (LVDP at 40 min: doxorubicin controls 55 ± 3%; doxorubicin with 10^{-5} *M* amifostine 68 ± 2%, $P = 0.05$; doxorubicin with 10^{-4} *M* amifostine 80 ± 3%, $P < 0.01$). The group receiving 10^{-5} *M* amifostine was intermediate between that treated with doxorubicin alone and that receiving doxorubicin associated with 10^{-4} *M* amifostine. Overall, 10^{-5} and 10^{-4} *M* amifostine appeared to decrease the acute toxicity of 2.5×10^{-5} *M* doxorubicin or to delay its onset; the LVDP decrease was significant at 40 min on treatment with doxorubicin alone and at 50 min on perfusion with 10^{-5} *M* amifostine and remained nonsignificant at 60 min on perfusion with 10^{-4} *M* amifostine as compared with controls (Fig. 2). Both doses of amifostine gave similar results with regard to +dP/dt, which differed from values noted for controls and for doxorubicin alone (Fig. 2).

Coronary flow and CK release

No significant change in coronary flow or oxygen uptake was observed when 2.5×10^{-5} *M* doxorubicin was added to amifostine after a 20-min stabilization period (Fig. 2). CK leakage did not significantly change when doxorubicin was perfused with any dose of amifostine (doxorubicin controls 3,071 ± 751 μIU/g, doxorubicin with 10^{-5} *M* amifostine 2,786 ± 578 μIU/g, doxorubicin with 10^{-4} *M* amifostine 2,573 ± 574 μIU/g).

Discussion

Overall, our results displayed an amifostine-induced increase in coronary flow along with a related decrease in oxygen extraction, with no change in inotropic function being seen. In addition, amifostine exerted a protective effect against acute doxorubicin cardiac toxicity, which appeared to be dose-related.

The increase in coronary flow induced by amifostine may be related to coronary dilation, since we observed no change in cardiac function or oxygen uptake. An increase in oxygen saturation previously described in humans [10] may be related to a vasodilator effect. However, the mechanisms of the coronary dilation we observed remains unclear. Amifostine alone had no significant effect on the inotropic heart function.

In a preliminary study, 10^{-5} *M* doxorubicin was not sufficient to induce acute cardiac toxicity in our experimental setting, unlike previously published data [17]. However, on this dose regimen the doxorubicin-induced LVDP decrease was significant after more than 60 min of perfusion, whereas doxorubicin was perfused for only 40 min in our study [17, 19].

The pattern of the acute cardiac toxicity induced by 2.5×10^{-5} *M* doxorubicin was similar to that described in previous reports [9, 19, 20]. When 10^{-5} or 10^{-4} *M* amifostine was perfused before and together with 2.5×10^{-5} *M* doxorubicin, we found significant changes in doxorubicin's toxicity, with a delayed and lesser decrease occurring in LVDP, +dPt, and −dPdt, demonstrating a protective effect of amifostine despite the small number of rats in each group.

Two main mechanisms have been considered to explain both acute and chronic doxorubicin-induced cardiotoxicity: reactive oxygen radical production and dysfunction of the sarcoplasmic reticulum, resulting in changes in intracellular calcium concentrations [9, 14, 15]. A direct interaction with reactive oxygen radicals, together with oxygen depletion, has been recognized as the mechanism responsible for the radioprotective activity of the aminothiol compounds [23]. This cardioprotective radical-scavenging activity of amifostine has also been demonstrated in an ischemia-reperfusion model [18]. Since the generation of free radicals may be an important mediator of doxorubicin-induced cardio-

toxicity [15], the protective effect we observed for amifostine against doxorubicin's toxicity may have been related to its radical-scavenging activity.

However, the sarcoplasmic reticulum calcium load may also be a major component of doxorubicin's cardiotoxicity [2, 4, 14]. Despite the coronary dilator effect, we did not observe inotropic changes, which may suggest amifostine-induced changes in myocardial calcium transient; also, in previous studies, amifostine did not appear to modify much of the epinephrine and norepinephrine pressor effect [6]. Some data favor a direct relaxation of vascular smooth muscle by WR-1065 [21].

The mean peak plasma concentration measured in humans after a 15-min infusion of 740 mg/m^2 was 2.35×10^{-4} M/l; the active concentrations of amifostine detected in our study were 10^{-4} and 10^{-5} M/l and appear to be readily achieved in the clinical setting [13].

The relevance of the model used to assess clinical cardiac toxicity is limited; discrepancies between cardiac toxicities encountered in clinical practice and those detected using this model with respect to epirubicin were explained by in vivo pharmacokinetic differences [19]. However, our aim was to search for cardiac protection when cardiac toxicity had been obtained; for this purpose we believe the model is sufficient as a first approach when doxorubicin is involved, since the acute and chronic cardiac toxicity of doxorubicin seem to share the same mechanisms, with chronic toxicity arising from repeated episodes of acute exposure inducing cumulative damage [17]. It may also be discussed whether amifostine or WR-1065 was the form responsible for our results. From previous in vitro data obtained on cultured myocardial cells it appeared that the use of amifostine or its active metabolite WR-1065 induced the same protective effects against doxorubicin, suggesting that amifostine is transformed into active WR-1065 by rat-heart myocytes [7].

In conclusion, the present study shows that in a Langendorff-type model of isolated perfused rat heart, 10^{-5} and 10^{-4} M amifostine alone increases the coronary flow, probably as the result of amifostine-induced coronary dilation. When associated with 2.5×10^{-5} M doxorubicin, a cardiotoxic concentration in our study, 10^{-5} and 10^{-4} M amifostine displayed a protective effect, which may be related to the known radical-scavenging activity of amifostine. In this study we did not address the issue of protection against chronic doxorubicin toxicity or of cardiac histology, which might be of help in assessing the extent of amifostine's protective effect. Further analysis of enzymatic, ionic, and energetic changes as well as free-radical production are also necessary for assessment of the mechanism of amifostine's action on cardiac function in this setting. Since amifostine is currently in use in clinical practice, a protective effect against the cardiac toxicity of doxorubicin may be of interest when high doses of anthracycline are needed.

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