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Pharmacological Determinants of the Antitumour Activity of Mitomycin C

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ABSTRACT. Recent investigations into bioreductive anticancer drugs have focused on profiling reductase enzymes and relating their expression to therapeutic activity in an approach referred to as enzyme directed drug development. However, few studies have attempted to validate this approach *in vivo* and even less is known about how the expression of reductases relates quantitatively and qualitatively to metabolic activation. In the present study, the antitumour activity, pharmacokinetics and metabolism of mitomycin C (MMC) has been determined *in vivo* in two murine adenocarcinomas of the colon, MAC 16 (high DT-diaphorase activity) and MAC 26 (low DT-diaphorase activity) after intra-tumoural injection of drug. Over a broad range of drug concentrations (50–250 μg), MAC 16 proved to be consistently the more sensitive tumour (e.g. 75 μg of MMC, T/C 11% for MAC 16 and 31% for MAC 26). Higher levels of parent drug (peak concentration 103 μg/tumour compared to 58 μg/tumour) were maintained over 45 min in MAC 16 after which time clearance was rapid from both tumours. Four metabolites were detected in both tumours characteristic of different pathways of metabolism. However, by far the major metabolite was 2,7-diaminomitosene (2,7-DM), an accurate indicator of metabolic activation of MMC. Despite higher reductase levels and greater sensitivity to the drug, there was 4-fold less production of 2,7-DM in MAC 16. These results indicate a lack of a simple relationship *in vivo* between reductase expression and metabolic activation and suggest factors other than pharmacological determinants being responsible for the chemosensitivity of the MAC tumours to MMC. BIOCHEM PHARMACOL **56**;11:1497–1503, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. mitomycin C; antitumour activity; MAC tumours; metabolic activation; pharmacokinetics; 2,7-diaminomitosene

Bioreductive anticancer drugs perhaps rank unique amongst cancer chemotherapeutic agents in that they offer the potential of selectivity against solid tumours through two possible mechanisms. First, by requiring anaerobic conditions to undergo metabolic activation before covalent binding to DNA [1], they may target the hypoxic cell fraction present in solid tumours known to be resistant to ionising radiation and conventional anti-neoplastic agents [2]. Second, because certain reductases such as DT-diaphorase (EC 1.6.99.2; NAD(P)H:quinone oxidoreductase) can be overexpressed in chemoresistant human tumours such as non-small cell lung cancer (NSCLC) and colon cancer in comparison to normal tissues [3–5], enhanced metabolic activation may occur in these tumours [6]. The latter approach has become known as enzyme directed rational drug design and requires the profiling of biopsy material for reductase enzyme activity in order to identify optimal patient groups to treat with bioreductive drugs [7]. For the enzyme directed approach to be successful, it obviously has to be established that a correlation holds between protein levels (expression and activity) and drug efficacy, not only *in vitro* in cells lines [8] but more importantly *in vivo* in solid tumours [6].

MMC‡ is the prototype bioreductive agent and, as well as being used as a therapeutic agent in the treatment of human cancer, has been employed extensively as a model compound to study bioreductive processes [9]. However, only a limited number of experiments have been performed *in vivo* with MMC in an attempt to validate the enzyme directed approach by correlating antitumour activity to the expression of bioreductase enzymes [10]. Such studies have produced contradictory results where both a positive (SCLC) and negative (gastrointestinal tract) correlation has been reported with DT-diaphorase in xenograft models [3, 11]. To date, no satisfactory explanation has been proposed to resolve these contradictory data. To further

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‡ *Abbreviations:* $AUC_{0-360\ min}$, area under concentration/time profiles; *cis*-hydro, 1,2-*cis* 1-hydroxy 2,7-diaminomitosene; DIC, dicoumarol; HPLC, high-performance liquid chromatography; i.t., intra-tumoural injection; MMC, mitomycin C; NCI, National Cancer Institute of America; NSCLC, non-small cell lung cancer; T/C, mean drug treated tumour volume/mean control tumour volume; 2,7-DM, 2,7-diaminomitosene; *trans*-hydro, 1,2-trans 1-hydroxy 2,7-diaminomitosene.

complicate matters, it now appears that the role of DT-diaphorase is much more complex and controversial than first believed where it has even been claimed to play a protective role in hypoxic cells [12, 13].

Little is known about the biologic fate of MMC in solid tumours despite the fact that metabolic activation is an integral component of its mechanism of action, partly due to analytical problems of detecting drug metabolites [14]. Lack of this information has particularly hampered progress in identifying pharmacological determinants of the *in vivo* antitumour activity of MMC and thus a proper evaluation of the predictive value of bioreductive enzyme determinations as indicators of chemoresponsiveness [15]. In the present study, the pharmacokinetics and metabolic fate of MMC in two solid tumours (MAC 16 and MAC 26), one high and one low in DT-diaphorase activity, has been determined and compared to both the antitumour activity of the drug in the two tumours and tumour levels of bioreductive enzymes [16].

MATERIALS AND METHODS

HPLC

All chromatographic analyses were carried out using a Hewlett-Packard (HP) Model 1090 liquid chromatograph equipped with a diode array detector (HP Analytical Instruments). System control, data acquisition and data analysis were performed using HP Chemstation software operating on a HP PC.

MMC was supplied by Kyowa Hakko Kogyo Co., through the auspices of Dr. John Kelly, Kyowa Hakko U.K. Standards of the major hydrolysis products of MMC, 1,2-*cis* 1-hydroxy 2,7-diaminomitosene (*cis*-hydro) and 1,2-*trans* 1-hydroxy 2,7-diaminomitosene (*trans*-hydro), were synthesised in house by treatment of MMC with 0.1 M of hydrochloric acid for 25 min at room temperature [17]. Standards of the major product of MMC monofunctional drug activation, 2,7-diaminomitosene (2,7-DM) and bifunctional drug activation, 10-decarbamoyl-2,7-diaminomitosene, were a kind gift from Professor Maria Tomasz, Department of Chemistry, Hunter College, New York, U.S.A.

Chromatographic conditions were as previously described [17]. The stationary phase was LiChrosorb RP-18 (7 μm particle size and 25 cm by 4 mm dimensions for the analytical column and 5 μm particle size and 4 mm by 4 mm dimensions for the guard column) supplied by Crawford Scientific. The mobile phase consisted of 10 mM sodium phosphate buffer pH 7.5 and methanol, 74:26 (HPLC grade, Rathburn Chemicals Ltd). Elution was isocratic at a flow rate of 1 mL/min and the column was maintained at 40°.

Extraction of Mitomycin C and its Metabolites from Tumour Tissue

Tumours (and plasma samples) for drug analysis, which were stored at −80°, were only partially thawed in ice prior to homogenisation of the whole tumour in 154 mM potassium chloride (1:2 w/v). A portion of homogenate (1 mL) was extracted twice with 5 mL of chloroform-propan-2-ol-ethyl acetate (2:2:1) [14]. The extracts were then evaporated to dryness under a stream of nitrogen gas. Residues were reconstituted in 300 μL of HPLC mobile phase and 100 μL was injected into the liquid chromatograph. Quantitation was by the external standard method where known quantities of MMC and its metabolites were added to control tumour homogenates and individual recoveries were determined, which was necessary since MMC and its metabolites exhibit different extraction efficiencies [14]. Chromatographic peaks were monitored at 360 nm for MMC and 310 nm for metabolites.

Antitumour Activity of Mitomycin C

The antitumour activity of MMC was determined in two murine adenocarcinomas of the colon referred to as MAC 16 and MAC 26 after i.t. injection. Breeding pairs of NMRI mice and the MAC tumours were kindly supplied by Professor J.A. Double and Dr. M.C. Bibby of the Clinical Oncology Unit, University of Bradford, UK. Mice were maintained under standard laboratory conditions of heating and lighting, fed a standard mouse diet (RM3(E): SDS) and given access to water *ad lib*. The tumours were propagated by serial s.c. passage of 1–3 mg of viable tumour via a trochar needle to animals weighing between 25 and 30 g. When tumours reached 0.02–0.1 cm^3, animals were randomised into groups of 8 and received either 50, 75, 125, 250, 500, or 1000 μg of MMC in an injection volume of 250 μL of sterile distilled water or drug vehicle (250 μL of distilled water) or no treatment. Approximately every 2 days, the mice were weighed and tumour volume (V) was calculated by measuring two diameters at right angles with callipers applying the formula $V = \pi/6 \times \text{length} \times \text{width}^2$. The antitumour activity of MMC treated groups compared to the drug vehicle control group (T/C) was calculated from tumour volumes for each dose of drug on Day 7 after approximately four tumour doublings.

In Vivo *Pharmacokinetics and Metabolism of Mitomycin C in the MAC Tumours*

Tumour bearing animals were randomised into groups of three and injected i.t. with 500 μg of MMC made up in a final volume of 250 μL of sterile distilled water. Animals were sacrificed at time 0, 1, 5, 15, 30, 45, 60, 120, and 360 min after drug administration, tumours were removed and immediately placed in liquid nitrogen. Blood was collected and immediately centrifuged at 15,000 *g* for 5 min in a microcentrifuge to separate plasma. All samples were stored at −80° for a maximum of 2 weeks prior to drug analysis.

Peak levels and time to peak levels of MMC and 2,7-DM were taken directly from concentration/time profiles. Area under concentration/time profiles ($AUC_{0-360\ min}$) was determined by the trapezoidal rule. Clearance was calculated

TABLE 1. Antitumour activity of Mitomycin C against s.c. growing murine adenocarcinomas MAC 16 and MAC 26 after intra-tumoural injection of drug

Dose of Mitomycin C (μg)	MAC 16 T/C (%)*	MAC 26 T/C (%)
50	18	29
75	11	31
125	12	23
250	8	17

*Antitumour activity was expressed as a percentage of the mean drug treated tumour volume/mean control tumour volume (T/C) on day 7 after approx. 3–4 tumour doublings.

from the formula dose/AUC. The *in vivo* metabolic conversion of MMC to 2,7-DM in both tumour types was calculated as the AUC ratio of 2,7-DM/MMC.

RESULTS AND DISCUSSION

Antitumour Activity of Mitomycin C against MAC 16 and 26

The antitumour activity of MMC was determined after i.t. injection of MMC and the T/C values at day 7 are presented in Table 1. Although the i.t. injection is not the normal route of administration for MMC, it has been shown to model closely the pharmacokinetics of the drug in rats [18], and to predict accurately the antitumour activity of the related bioreductive drug indoloquinone EO9 in a panel of 4 solid tumours including MAC 16 and 26 [19]. In addition the i.t. route has enabled metabolic processes to be studied *in vivo* in solid tumour tissue, which hitherto had proved inaccessible to investigation [20, 21] due to the rapidity of enzyme catalysed biotransformation of MMC and its primary metabolites [22, 23].

Over a wide range of drug concentrations (50–250 μg), MAC 16 proved to be consistently more sensitive to MMC than MAC 26. At the highest concentrations studied (500 and 1000 μg), it was not possible to obtain full growth curves due to significant drug induced toxicity. When compounds are administered i.p. MAC 16 is normally less sensitive than MAC 26 to cytotoxic drugs, particularly alkylating agents such as cyclophosphamide, melphalan, TCNU and mitozolamide [24, 25], with notable exceptions being bioreductive drugs (MMC, indoloquinone EO9) and doxorubicin [26]. In general, MAC tumours are comparatively chemoresistant and coupled to their unique biologic properties have been claimed to be an excellent model for human colon cancer [27]. Thus, the results obtained in the present study with i.t. injections of drug are consistent with those obtained with MMC and other bioreductives (EO9) after systemic drug administration.

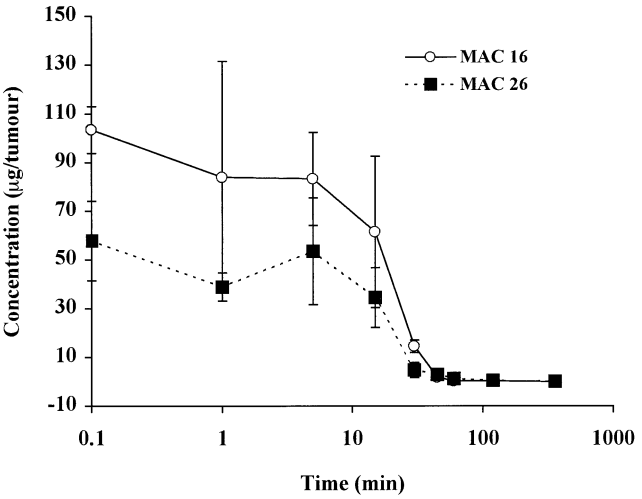

FIG. 1. Concentration time profiles of mitomycin C in MAC 16 and MAC 26 tumours of NMRI mice after i.t. injection of 500 μg of mitomycin C. Each value represents the mean ± SE from N = 3 animals.

Pharmacokinetics of Mitomycin C in the MAC Tumours

Tumour pharmacokinetic parameters derived after i.t. injection of MMC are summarised in Table 2. The main difference between the two tumours was that in MAC 16 significantly higher concentrations of the parent drug were maintained in the tumour over 45 min. After this time period drug levels fell rapidly to close to zero in both tumours (see Fig. 1). In the plasma of MAC 16 bearing mice, MMC concentrations accumulated more slowly to their peak level: 30 min compared to 5 min for MAC 26 (Fig. 2). By 2-hr plasma levels in both groups were close to

TABLE 2. Pharmacokinetics of mitomycin C and 2,7-diaminomitosene in MAC 16 and MAC 26 tumours and plasma after i.t. injection of 500 μg of drug

	MAC 16				MAC 26			
	Mitomycin C		2,7-DM		Mitomycin C		2,7-DM	
Parameter	Tumour	Plasma	Tumour	Plasma	Tumour	Plasma	Tumour	Plasma
Peak level (μg/tumour)	103	15.0	5.9	ND†	57.8	15.2	5.1	ND
Time to peak (min)	0.1	30	0.1	ND	0.1	5	5	ND
$AUC_{0\text{-}360min}$ (μg × hr)	319	130	19.6	ND	196	88.1	49.9	ND
*2,7-DM: MMC ratio		0.06				0.26		
Clearance (mL/hr)		3.9		ND		5.7		ND

*Ratio of the tumour AUC 2,7-DM/MMC.
†ND, not detected.

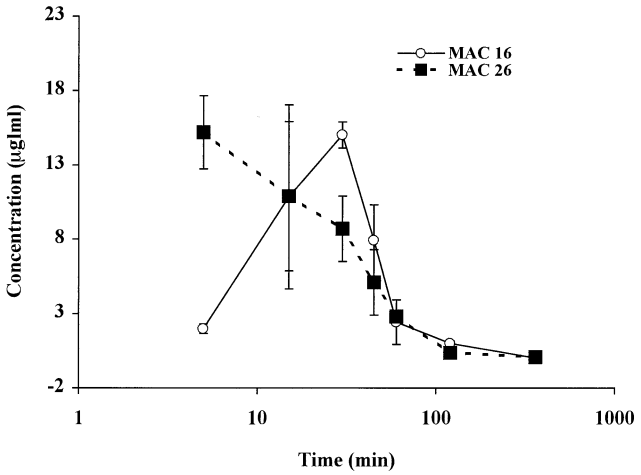

FIG. 2. Concentration time profiles of mitomycin C in the plasma of NMRI mice bearing MAC 16 and MAC 26 tumours after i.t. injection of 500 µg of mitomycin C. Each value represents the mean ± SE from N = 3 animals.

baseline values. These data are consistent with the short half life of 20–28 min reported for elimination of MMC from plasma and tissues of rats and mice after systemic drug administration [20, 28, 29].

MAC 16 and MAC 26 possess markedly different histology with the former being a poorly differentiated, poorly vascularised, highly necrotic tumour that induces cachexia and the latter being well differentiated and well vascularised [24]. The histology of both of these tumours was confirmed in the present study [16]. It is conceivable that after i.t. injection MMC concentrations were higher in MAC 16 due to drug retention in necrotic, poorly vascularised areas of the tumour. This may also explain the delayed appearance of the drug in plasma. Whereas, in MAC 26 which is well vascularised, tumour drug levels were lower due to more rapid transit into the systemic circulation.

Metabolic Activation of Mitomycin C in the MAC Tumours

Four metabolites of MMC were detected in both the MAC 16 and MAC 26 tumours after i.t. injection of the drug (data not shown). These were identical to the same four products detected *in vitro* in incubations of MMC with MAC 16 and MAC 26 homogenates [16]. Two were identified as the hydrolysis products *trans*-hydro and *cis*-hydro but were only minor products [22, 30]. A minor peak corresponding to the isomeric form of the parent drug was detected in samples [17, 31]. By far the major metabolite detected in the tumours was 2,7-DM, which is not generated through chemical degradation of MMC but is a genuine product of reductive metabolism [32]. The fourth and final metabolite detected was attributed to decarbamoyl 2,7-DM, a secondary metabolite of MMC and a product of bifunctional metabolism of the drug [22]. It was only detected sporadically in samples at concentrations close to the detection limit of the HPLC assay.

Identification of 2,7-DM in the MAC tumours was considered of great significance since this metabolite is viewed as an important and accurate biologic marker of MMC metabolic activation [15]. 2,7-DM is now believed to be evolved as an intermediate during pathways of biotransformation that result in the formation of more cytotoxic bifunctional DNA adducts such as interstrand crosslinks [10, 33, 34]. In its own right, 2,7-DM can act as a substrate for metabolic activation leading to monoalkylation of the major groove of DNA [35]. Its appearance has been shown to correlate to the cytotoxicity of MMC in colon cancer cells *in vitro* [36] and to the antitumour activity of MMC against the MAC 16 tumour *in vivo* [37]. In addition, the kinetics of its generation in solid tumours follows the time scale for covalent modification of DNA in cancer cells [18].

The concentration time profiles and pharmacokinetics of 2,7-DM in MAC 16 and MAC 26 are reported in Fig. 3 and Table 2, respectively. 2,7-DM, or to that matter, any other metabolite of MMC was not detected in plasma. When levels of the parent drug were taken into account, a fourfold greater conversion of MMC to 2,7-DM occurred in MAC 26 (Table 2) through sustained production rather than by increased intensity of formation (Fig. 3).

The MAC 16 tumour possesses at least three sources of MMC reducing activity located in different subcellular compartments including cytosolic DT-diaphorase, microsomal NADPH cytochrome P-450 reductase and a group of mitochondrial one-electron reductases [16]. MAC 26 possesses only one source of MMC reducing activity located in mitochondrial fractions. Therefore, one might expect MAC 16 to exhibit increased capacity over MAC 26 to metabolise MMC. However, in a previous study when MMC was added to tumour homogenates much lower concentrations of 2,7-DM were detected in the MAC 16 incubations [16]. A mechanism was proposed to explain

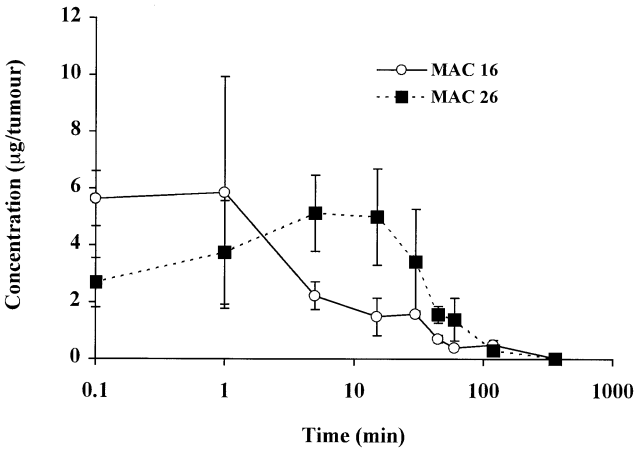

FIG. 3. Concentration time profiles of 2,7-diaminomitosene in MAC 16 and MAC 26 tumours of NMRI mice after i.t. injection of 500 µg of mitomycin C. Each value represents the mean ± SE from N = 3 animals.

this result involving re-interpretation of the role of DT-diaphorase in MMC metabolic activation [10, 15, 16]. When DT-diaphorase is overexpressed in a cell line or tumour like MAC 16 it acts as the predominant reductase, but it is less efficient at metabolising the drug due to substrate inhibition [33, 38]. Thus, in cell lines rich in DT-diaphorase, the activity of MMC is reduced comparatively due to the fact that metabolic activation of the drug is constrained [12, 13]. However, this argument holds only in hypoxic cells when DT-diaphorase is actively competing against more efficient one-electron reductases [33]. Under aerobic conditions increased expression of DT-diaphorase results in greater cytotoxicity since only metabolism by this enzyme results in metabolic activation as one electron reductases are inhibited by molecular oxygen [39–41]. The present study has shown, for the first time, that metabolic activation of MMC - as determined by the appearance of 2,7-DM - is also reduced *in vivo* in a tumour high in DT-diaphorase (15–23 fold greater than MAC 26) [16]. In addition, it would appear from this pattern of metabolism that reduction of MMC is occurring in the MAC tumours predominately under hypoxic conditions.

These conclusions may shed some light on previously published *in vivo* studies where both a positive and inverse correlation was observed between DT-diaphorase expression and antitumour activity [3, 11, 42]. Taking the case of the negative correlation first, in the gastrointestinal tract xenografts containing the higher levels of DT-diaphorase, according to the above argument, the degree of MMC metabolic activation (under hypoxic conditions) would decrease as more metabolism is transferred from higher efficiency one electron reductases to lower efficiency DT-diaphorase. Thus, antitumour activity would also decrease, as reported [11]. Interestingly, co-administration of DIC, an inhibitor of DT-diaphorase, increased antitumour activity in the tumour panel but the effect was more pronounced in the xenografts that expressed the highest levels of DT-diaphorase [11]. By eliminating competition from diaphorase DIC may actually act as a promoter of metabolic activation. Co-treatment of hypoxic EMT6 mouse mammary tumour cells with MMC and DIC resulted in an increase in both intra- and inter-strand DNA crosslinks [43]. In the study where a positive correlation was observed between DT-diaphorase and antitumour activity, the relationship was not absolute. Two from eight xenografts with elevated levels of DT-diaphorase did not respond to MMC, suggesting factors other than bioreductive enzyme expression being responsible for activity [3].

The main finding of the present study was the lack of a correlation *in vivo* between reductase enzyme expression, MMC metabolic activation and antitumour activity. The enzyme directed approach to bioreductive drug development relies on this relationship to hold in order to be of prognostic value [6]. Such a relationship has been demonstrated with DT-diaphorase and MMC in the NCI disease-orientated panel of cell lines [8]. However, these experiments were performed in aerobic cells. In this situation it is not unrealistic to expect a positive correlation because one-electron reductases like cytochrome P-450 reductase are inhibited and only a two electron reductase like DT-diaphorase is capable of metabolic activation [44, 45]. However, more recent experiments with xenografts suggest that metabolic activation of MMC occurs *in vivo* predominately under hypoxic conditions [11, 42]. Clearly, *in vitro* MeDZQ studies do not take into account a number of physiological factors that can have a major bearing on activity such as pH, pharmacokinetics, oxygenation and cellular heterogeneity [46]. Recent *in vivo* studies with related bioreductive drugs indoloquinone EO9 and MeDQZ (2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone), have shown a complete lack of a correlation between expression of a single bioreductive enzyme (including cytochrome P-450 reductase, DT-diaphorase and cytochrome b5 reductase) and antitumour activity [19, 47]. Nonetheless, both of these compounds are much better substrates for DT-diaphorase than MMC and their *in vitro* activity correlates impressively to the expression of this enzyme [48–50]. Therefore, there is accruing evidence that only a poor relationship exists between bioreductive enzyme expression and *in vivo* antitumour activity of bioreductive drugs [10].

The lack of correspondence between apparent metabolic activation of MMC and antitumour activity indicates factors other than primary DNA damage (crosslinking) may be more critical in defining chemosensitivity in the MAC tumours. Apart from the physiological variables described above, which also have a major bearing on which reactive intermediates and DNA adducts are formed [34], more recently a number of cellular and molecular determinants of MMC activity have been identified. These are activated downstream of DNA covalent modification and are not necessarily dependent on the level of DNA damage [51]. They include activation of $p56/p53^{lyn}$ and phosphorylation of $p34^{cdc2}$ leading to G2/M blockade at the mitotic cell cycle checkpoint [52] and promotion or inhibition of entry into apoptosis [51]. Identification of these cellular factors *in vivo* represents the next stage in our understanding of the determinants of the antitumour activity of MMC.

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