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Inhibition of human glutathione transferases by multidrug resistance chemomodulators *in vitro*

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Abstract

Reversal of the drug-resistance phenotype in cancer cells usually involves the use of a chemomodulator that inhibits the function of a resistance-related protein. The aim of this study was to investigate the effects of MDR chemomodulators on human recombinant glutathione S-transferase (GSTs) activity. IC_{50} values for 15 MDR chemomodulators were determined using 1-chloro-dinitrobenzene (CDNB), cumene hydroproxide (CuOOH) and anticancer drugs as substrates. GSTs A1, P1 and M1 were inhibited by O^6-benzylguanine (IC_{50}s around 30 μM), GST P1-1 by sulphinpyrazone (IC_{50} = 66 μM), GST A1-1 by sulphasalazine, and camptothecin (34 and 74 μM respectively), and GST M1-1 by sulphasalazine, camptothecin and indomethacin (0.3, 29 and 30 μM respectively) using CDNB as a substrate. When ethacrynic acid (for GST P1-1), CuOOH (for A1-1) and 1,3-bis (2-chloroethyl)-1-nitrosourea (for GST M1-1) were used as substrates, these compounds did not significantly inhibit the GST isoforms. However, progesterone was a potent inhibitor of GST P1-1 (IC_{50} = 1.4 μM) with ethacrynic acid as substrate. These results suggest that the target of chemomodulators in vivo could be a specific resistance-related protein.

Keywords: *Glutathione S-transferases, P-glycoprotein, chemomodulators, multidrug resistance, O^6-benzylguanine, BCNU, inhibition*

Introduction

During treatment of many cancers, there is often development of drug resistance in a tumour that was originally sensitive to treatment resulting in a phenomenon known as multidrug resistance (MDR) [1]. Many mechanisms are involved in MDR and these include alterations in drug transport resulting in impaired entry or enhanced efflux of the drug from the tumour cell, enhanced DNA repair, alterations in target proteins and alterations in drug metabolism [2]. The detoxifying enzymes that are involved include glutathione-dependent enzymes, glutathione transferases (GST EC 2.5.1.18) and glutathione peroxidase [3]. The major cytosolic isoforms of glutathione transferases (GSTs) are grouped into the Alpha (A), Mu (M), Pi (P), Omega (O), Sigma (S), Theta (T) and Zeta (Z), classes according to structural and catalytic properties [4]. GSTs detoxify xenobiotics that include several carcinogens, mutagens, antibiotics, antiparasitic and anticancer drugs and these electrophiles are reduced to less toxic metabolites by this conjugation reaction [5]. These conjugates are then pumped out of the cell by several membrane bound proteins including MRP1 and P-glycoprotein [6]. MRP1 has been identified as a glutathione export pump and since GST conjugation reactions results in the formation of glutathione conjugates, there is a possibility of MRP1 and GST working together to give resistance to anticancer compounds. Thus, GST A1-1 is required for MRP1-associated resistance to chlorambucil in MCF7 cells [7,8].

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Drug resistance in cancer cells can be overcome by administration of non-toxic reversing agents called chemomodulators or enhancers together with the anti-cancer agents [9] resulting in a reversal of the MDR phenotype. P-glycoprotein activity can be down regulated by use of inhibitors, which include calcium channel blockers such as verapamil and the immuno-suppressive agent cyclosporine [10]. The DNA repair enzyme methylguanine methyl transferase (MGMT) is a chemoprotective enzyme that removes mutagenic and cytotoxic alkyl adducts (i.e. chloroethyl groups) from the O^6-postion of DNA –guanine and prevents the formation of DNA interstrand cross links (ISCs)[11]. The lethal lesions induced by chloroethylating agents such as 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) can be removed by MGMT and O^6-benzylguanine (O^6-BG) is used for chemomodulation of this enzyme. Since GSTs are involved in the metabolism of BCNU [12], we postulated that in the reversal of the resistance phenotype, O^6-BG could also be affecting the function of GSTs. In cases where GSTs are thought to play a role in drug resistance, chemomodulation of therapy could involve countering the increased GST activity. This might be achieved by using inhibitors of glutathione synthesis e.g. buthionine sulphoxime or by using GST inhibitors such as ethacrynic acid [13]. Few GST inhibitors that are active *in vivo* have been developed, but a number of inhibitors have been reported that are suitable for *in vitro* studies. Among these are sulphasalazine, indomethacin, curcumin, haloenol lactone, some antimalarials and organotin compounds [5,9,14].

The aim of this study was to investigate the effects of MDR chemomodulators on human recombinant glutathione S-transferase (GSTs) activity in the context of searching for effective and clinically acceptable inhibitors which would provide a combined modulation of anticancer drug-resistance at the levels of GST-mediated conjugation and any other MDR-related mechanism Figure 1.

Materials and methods

Materials

Most chemicals including ethacrynic acid (ETA) were purchased from Sigma (St. Louis, MO, USA) and BCNU (Becenum™) was obtained from Bristol-Myers Squib (Stockholm, Sweden). *E. coli* clones expressing human recombinant GSTs were a kind donation from Prof. Bengt Mannervik (Department of Biochemistry, Biomedical Centre, Uppsala University, Sweden).

Heterologous expression and purification of recombinant human glutathione transferases

Glutathione transferase A1-1, M1b-1b, and P1-1, were expressed in *Escherichia coli* and prepared as described before [15]. Affinity chromatography purification of GSTs was carried out on glutathione -Sepharose 4B or S-hexylglutathione-Sepharose 6B affinity matrix. The eluted protein was concentrated using a PLGC membrane NMLW 10000 (Pharmacia Biotech, Uppsala, Sweden). The concentrated protein was dialysed against 10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 0.2 mM dithiothreitol, and 0.02% w/v NaN_3. The protein concentration was determined by the method of Lowry et al. [16]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using purified GST fractions on 15% slab gels as described by Laemmli [17] using a Biorad Protean electrophoresis system. Protein bands were stained with Coomassie Blue-G. Analytical isoelectric focusing was carried out on precast gels (pH 3-10) following the manufacturer's instructions (Pharmacia Biotech, Uppsala).

Assay of GST activity

Enzyme activity was assessed by measuring the conjugating activity with 1-chloro-2, 4-dinitrobenzene (CDNB), and ethacrynic acid (ETA) [18], and the denitrosation of BCNU [19]. The assay with CDNB was adapted so that absorbance was read in a SpectraMax 340 ELISA plate reader (Molecular Devices, California, USA) whilst activity with ETA was determined using a Shimadzu UV spectrophotometer, UV1601 (Shimadzu Corporation, Tokyo, Japan). The cumene hydroperoxide is reduced in a GST-oxidised glutathione (GSSG)-reductase coupled assay and the rate of formation of $NADP^+$ from NADPH is measured at 340 nm [20]. The effects of MDR inhibitors were also investigated on the inactivation of the anticancer drug BCNU by GST M1-1. The formation of nitrite was used as an index of BCNU denitrosation. Conditions for enzymatic assay were as described by Berhane et al. [12].

Inhibition by MDR chemomodulators

For the determination of the concentration of inhibitor at which 50% inhibition of enzyme activity was obtained (IC_{50}), the reaction mixture contained 15 μL of the chemomodulator dissolved in either phosphate buffer (0.1 M, 1 mM EDTA) or 95% ethanol in a final reaction mixture of 300 μL. The total organic solvent concentration was less than 5%. The ETA conjugation reaction for GST P1-1 was initiated by addition of 25 μL ETA (8 mM in ethanolic solution) and 50 μL GSH (5 mM in buffer) in a 1000 μL reaction mixture. The final concentration of ethanol was less than 5%. The blanks contained all the components except for GST. The effects of MDR inhibitors on GST A1-1 were further investigated with cumene hydroperoxide as a substrate. The IC_{50} values were determined from plots of percent control activity versus log inhibitor

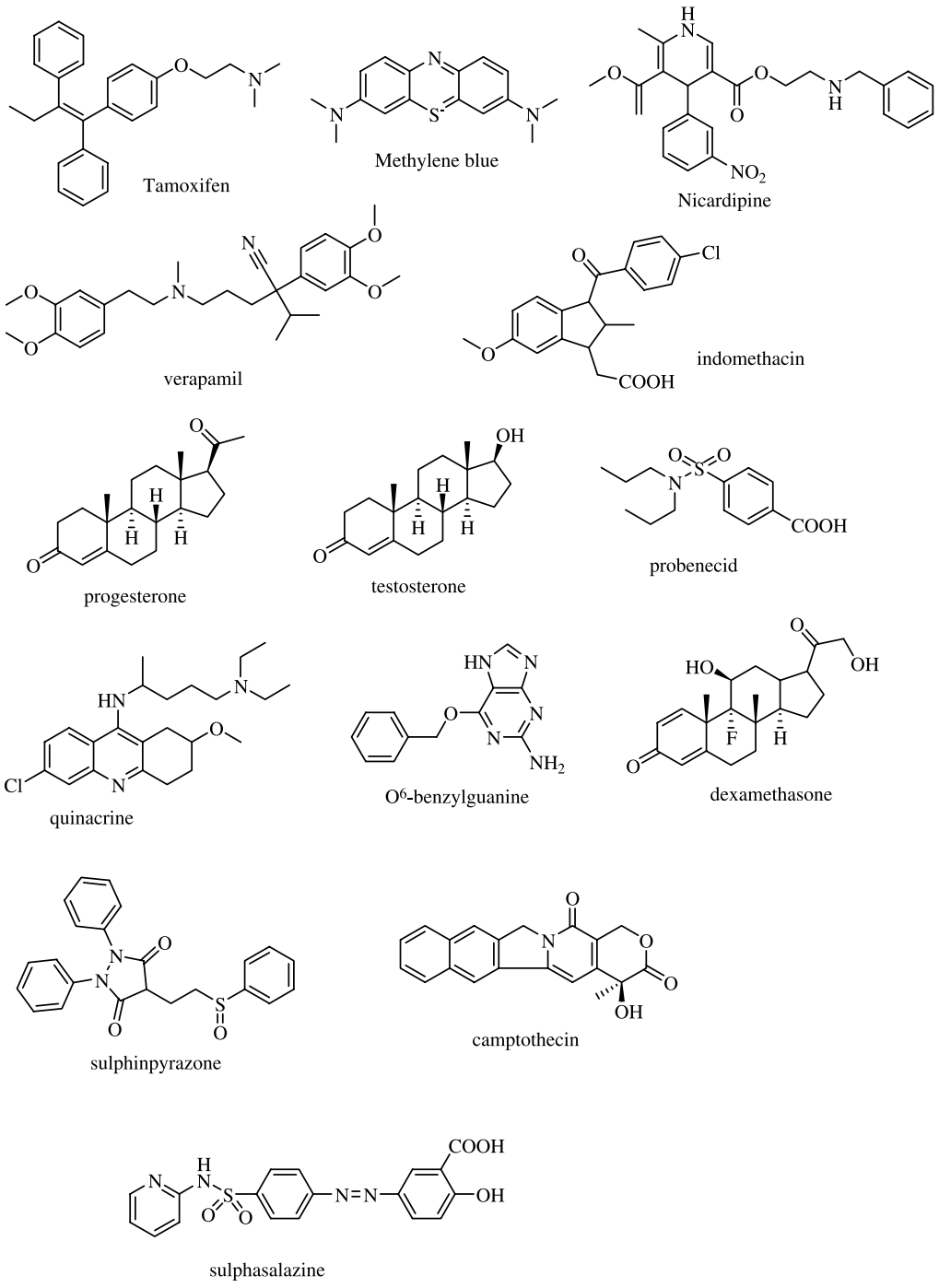

Figure 1. The structure of MDR-chemomodulators used in this study.

concentration as analysed using computer software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA).

Results

Purification and characterisation of heterologously expressed GSTs

Human GSTs heterologously expressed in *E. coli* were A1-1, M1 (allelic variant b) and P1-1 (allelic variant a). These were purified by affinity chromatography. The GSTs were purified to homogeneity and a single band was obtained on SDS-PAGE analyses (data not shown). Specific activities of 84, 190, and 129 μmoles/min/mg protein were obtained for GSTs A1-1, M1b-1b, and P1-1 respectively using CDNB as the substrate. The effect of standard inhibitors on GST activity was also determined. The inhibition characteristics (IC_{50} values) of recombinant GSTs from *E. coli* were comparable with those reported previously [21] (data not shown).

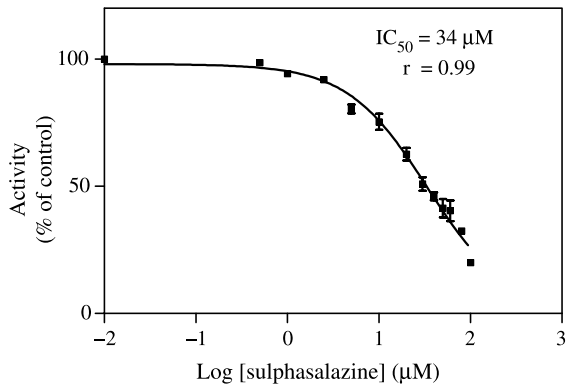

Figure 2. Inhibition of GST A1-1 by sulphasalazine using CDNB as the substrate. The IC_{50} value is inhibitor concentration giving 50% inhibition of the enzyme activity in the standard assay system with 1 mM 1-chloro-2, 4-dinitrobenzene and is obtained from plots of percent activity versus log inhibitor concentration (Graphpad Prism^{TM}-Software).

substrates for each of the GSTs. Data from such graphs are shown in Table I. Some compounds inhibited only one isoform, or inhibited all isoforms but with high potency for one isoform e.g. sulphasalazine had high inhibition potency for GST M1-1. Some of the compounds had no effect at all and these include nicardipine, testosterone, quinacrine, dexamethasone, methylene blue, tamoxifen, verapamil and probenecid. Other compounds were effective inhibitors of all the three isoforms e.g. O^6 benzylguanine inhibited all three isoforms; A1-1, M1-1 and P1-1. It is important to note that those compounds that were inhibitors with CDNB as a substrate did not inhibit or showed decreased inhibition when other substrates were used (Figure 3). Progesterone, however, showed no inhibition when CDNB was used as substrate but, with ethacrynic acid, it was found to be a potent inhibitor for GST P1-1.

Effects of MDR inhibitors on human recombinant GSTs

The effect of MDR inhibitors on the activity of human recombinant GST activity was assessed by measuring the conjugating activity with 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETA) for P1-1, cumene hydroperoxide (CuOOH) for A1-1 and BCNU for GST M1-1. Figure 2 shows a typical plot used to determine the IC_{50} value for sulphasalazine. Similar graphs were plotted to determine IC_{50} values for each MDR modulator using CDNB and other

Discussion

MDR modulators have a variety of structures but most importantly these agents also possess different mechanisms of action for the reversal of MDR. Most of these agents interact directly with Pgp or other protein pumps e.g. MRP, but some may sensitise drug-resistant tumour cells independent of Pgp [10]. The plurality in the mechanism of action of the chemomodulators offers a possibility to reduce the number of modulators during adjunct chemotherapy

Table I. IC_{50} values (μM), of human recombinant glutathione S-transferases for MDR reversing agents.

	GST isoform						
Modulator	A1-1	M1-1	P1-1	Plasma Concentration[#]			
			IC_{50} CDNB [μM]*				
Sulphinpyrazone-	NI	NI	69	2000 μM			
Camptothecin	74	29	>100	–			
Progesterone	NI	NI	NI)	5 μM			
Sulphasalazine	34	0.3	NI	30 μM			
Indomethacin	160	30	390	100-200 μM			
O^6-benzylguanine	26	28	24	10 μM			
	IC_{50}^{CuOOH}	IC_{50}^{BCNU}	IC_{50}^{ETA}	K_m (μM)[§]			
				CDNB	CuOOH	ETA	BCNU
Sulphinpyrazone	NI[¶]	NI	NI	(P1-1)	(A1-1)	(P1-1)	(M1-1)
Camptothecin	>100	–	NI	1200	–	29	1800
Progesterone	NI	NI	1.4				
Sulphasalazine	66	>10	NI				
Indomethacin	NI	>100	NI				
O^6-benzylguanine	NI	NI	NI				

*The IC_{50} value is inhibitor concentration giving 50% inhibition of the enzyme activity in the standard assay systems with 1 mM 1-chloro-2,4-dinitrobenzene and ethacrynic acid was obtained from plots of percent activity versus log inhibitor concentration as shown in Figure 2. Activities were determined with CDNB in quadruplicate in a SpectraMax 340 ELISA reader equipped with the kinetics mode and with ETA in a Shimadzu UV 1601 spectrophotometer. [#]Plasma concentration during reversal of MDR were obtained from Ferry et al. [22], Draper et al. [27] and Dolan et al. [11] [§]K_m values for CDNB and ETA for GST P1-1 were obtained from Johansson et al. [31], whilst that for BCNU with rat GST M was obtained from Smith et al. [32]. [¶]NI no inhibition observed within the concentration range of 0-100 μM.

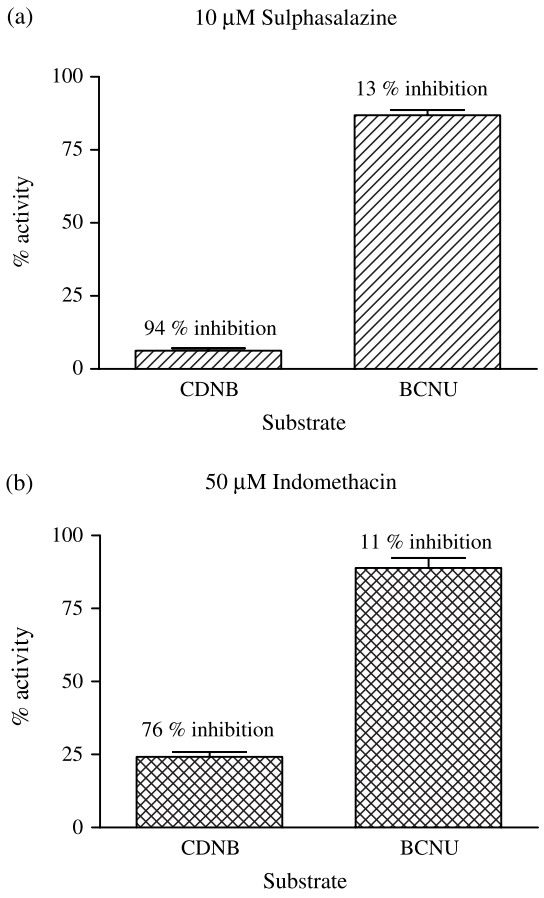

Figure 3. The effects of 10 μM sulphasalazine (a) and 50 μM (b) indomethacin on GST M1-1 activity with CDNB and BCNU as substrates. CDNB data are means + SD of quadruplicate experiments each performed 3 times. BCNU data are the means + S.D. of duplicate experiments.

since one compound would simultaneously target more than one resistance-related protein. It is important to identify the range of effects of MDR chemosensitisers, because there is co-expression of several resistance mechanisms in the tumour cell and they may be affected by a single agent in the process of reversing MDR [2]. This study has shown that some MDR chemomodulators are potent inhibitors of human recombinant GSTs with IC_{50} values between 0.3 and 400 μM with CDNB as a substrate although generally below 100 μM. Of the 6 MDR modulators noted to affect activity when using CDNB as a substrate, sulphasalazine was the most potent inhibitor.

Considering the IC_{50} values relative to the expected plasma concentration of the drugs in the body during clinical trials of reversing MDR [22], our results suggest that some MDR inhibitors may be effective inhibitors of GSTs *in vivo* and, hence, could provide combined modulation of more than one resistance-related protein. Sulphasalazine may be an effective inhibitor of GST M1-1 *in vivo* since the IC_{50} value obtained for this drug using the substrates CDNB and glutathione (GSH) was well below the plasma concentrations of this drug when used clinically [2]. Sulphinpyrazone inhibited GST P1-and is also known to inhibit the function of MRP 1 and, therefore, may be serve as a good candidate for combined modulation of GST activity in cells overproducing GST P1-1 and the drug efflux pump MRP1. In contrast, the IC_{50} values for O^6-benzylguanine are above the value of the plasma concentration expected during chemomodulation in cancer patients exhibiting high MGMT expression (Table I) and this drug, therefore, is not likely to be useful clinically as a dual modulator of GST and MGMT activity.

Chemomodulation during chemotherapy could involve targeting GSTs responsible for the breakdown of several alkylating agents and GSTs have been suggested to be necessary for the efflux of anti-cancer drugs from tumour cells [23]. Studies of resistance to chlorambucil in MCF7 breast carcinoma cells showed that GSTs (A1, P1) and MRP1 acted in synergy to protect cells from the cytotoxicity of chlorambucil and ethacrynic acid respectively [8]. These proteins may present the hydrophobic ligands to efflux pumps either conjugated to GSH or non-conjugated [24].

Most of the compounds used in this study did not show inhibitory effects towards GSTs, suggesting that the target of these compounds *in vivo*, are particular resistance-related proteins in the cell. Methylene blue for instance reverses MDR by inhibiting Pgp function [25], but, as this study has shown, it has no effects on GSTs. Whilst indomethacin is reported to increase the cytotoxicity of cisplatin in cancer cell lines [26], the mechanism of action could be solely due to inhibition of export of the drug by MRP and not inhibition of the conjugation of the drug to GSH as catalysed by GSTs [27].

Although most studies of inhibition of GST activity in normal and tumour samples use the electrophilic substrate CDNB, this is a non-drug substrate and the question remains as to the suitability of this substrate for clinical investigations. For instance, we have shown that the potency of antimalarials as inhibitors of human recombinant GSTs was substrate-dependent [14]. In another study Zhang and Das [28], showed that the inhibitory potency of tannic acid on rat liver GSTs was also substrate dependent. Sulphasalazine was shown to be a potent inhibitor of both GST A1-1 and M1-1 *in vitro*, using CDNB as a substrate but as shown in Figure 3, the inhibition potential decreased when the alkylating agent BCNU was used as a substrate for GST M1-1. It is interesting to note that when ETA was used as a substrate, an IC_{50} value for progesterone was obtained (1.4 μM) whilst there was no inhibition obtained when CDNB was used as a substrate. This illustrates the usefulness of using other substrates in addition to CDNB. The most appropriate substrate for inhibition of GSTs would be the anti-cancer drug that is a substrate of the enzyme.

Many compounds have been shown to inhibit GSTs *in vitro* but only few compounds have been identified which are active inhibitors *in vivo* [29]. It is still necessary to carry out studies *in vitro*, because such studies will identify which compounds are potential inhibitors. In this respect our study demonstrates that it is important to use not only CDNB but other relevant substrates such as the alkylating anticancer agents that have been shown to be substrates of these enzymes. No firm conclusions with regards to potential efficacy and selectivity of inhibitors would be derived from using CDNB alone as the substrate in inhibition studies.

It is therefore recommended that investigations of inhibition of GST activity in possible reversal of alkylating anticancer drug resistance make use of a drug-substrate i.e. the particular alkylating agent being used to treat the particular cancer rather than CDNB. Further work needs to be done to evaluate the clinical use of GST inhibitors as reversal agents in resistance to alkylating agents during cancer treatment. There is need to determine whether these compounds inhibit other resistance-related proteins like glutathione peroxidase, topoisomerase II and DNA repair enzymes. In addition further work needs to be carried out to evaluate the effects of second and third generation MDR inhibitors on GSTs since there is evidence of the role of synergy' between these enzymes and drug transporters in anticancer drug resistance [30].

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