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Original Paper

Cytotoxic Activity of Calcein Acetoxymethyl Ester (Calcein/AM) on Primary Cultures of Human Haematological and Solid Tumours

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The aim of this study was to determine the *in vitro* cytotoxicity of calcein acetoxymethyl ester (Calcein/AM) on primary cultures derived from solid and haematological human tumours. Calcein/AM is a fluorescent dye that localises intracellularly after esterase-dependent cellular trapping and which has shown cytotoxic activity against various established human tumour cell lines at relatively low concentrations. The semi-automated fluorometric microculture cytotoxicity assay, based on the measurement of fluorescence generated from cellular hydrolysis of fluorescein diacetate to fluorescein, in microtitre plates was used for the evaluation of Calcein/AM activity in tumour cell suspensions from patients. The cytotoxicity was measured as a survival index (SI), defined as the fluorescence as a percentage of control cultures. A total of 163 evaluable samples from various tumours were tested with continuous drug exposure. The activity of Calcein/AM was compared with representatives of six major classes of standard chemotherapeutic drugs. Calcein/AM was found to induce concentration-dependent decreases in the SI of both haematological and solid tumour cells. The ratio of solid over haematological tumour activity increased at a rate that was concentration dependent. Although it was relatively less active than cisplatin against solid tumours, Calcein/AM showed higher solid tumour activity compared to leukaemic specific agents (cytarabine and amsacrine), vincristine and doxorubicin (Dox). Among the solid tumours tested, childhood tumours, non-small cell lung cancer and sarcomas were the most sensitive to Calcein/AM. The best correlation between SI values was seen between Calcein/AM and Dox, with weaker correlations to representatives of antimetabolites, platinum compounds, topoisomerase II inhibitors, tubulin interactive agents and alkylators. Non-cytotoxic concentrations of cyclosporin A significantly potentiated calcein-induced cytotoxicity. The results show that Calcein/AM is differentially active against haematological tumours, but with substantial activity against solid tumours. The drug may represent a new class of anticancer compound with a unique means of drug delivery.

Key words: drug screening, drug sensitivity assay, Calcein/AM, antitumour activity
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INTRODUCTION

CALCEIN/AM (4′5′-bis(*N*,*N*-bis (carboxymethyl) aminomethyl) fluorescein acetoxymethyl ester) is a non-fluorescent fluorescein analogue that can rapidly enter cells and is hydrolysed to free, strongly fluorescent calcein by cytoplasmic esterases [1, 2]. Free calcein is well retained by viable cells with intact plasma membranes, and has an apparent cytoplasmic distribution pattern [1, 2]. Calcein/AM has been mainly used as a cytoplasmic marker and viability probe [1, 3–5].

Recently, it has been shown that the AM ester of calcein is probably a substrate for the P-glycoprotein (Pgp) associated with multidrug resistance (MDR), and may be used as an easily operated functional fluorescent probe for this drug efflux protein [5–7]. However, after further evaluation *in vitro* of this molecule, it became apparent that Calcein/AM also showed cytotoxic activity alone against several cell lines at relatively low concentrations [8]. In common with most established cytotoxic drugs, the pattern of induced cell death was indicative of apoptosis [8]. Based on these observations, we decided to undertake further exploration of this *in vitro* activity before starting *in vivo* testing in tumour-bearing animal models.

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In vitro cytotoxic drug response patterns observed in primary cultures of human tumour cells have been previously shown to mimick to a large extent the clinical activity pattern of standard drugs [9, 10]. Thus, tumour-type specificity determined in primary cultures of tumour cells from patients may be an important predictor for the potential clinical utility of cytotoxic drugs. The present investigation was therefore undertaken to study the efficacy of Calcein/AM on primary cultures of human tumour cells representing both haematological and solid tumours, comparing it with some standard anticancer drugs.

MATERIALS AND METHODS

Tumour samples

163 (80 haematological and 83 solid) tumour cell samples, obtained from patients undergoing routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling, were successfully analysed. The number of each tumour type is listed in Table 1. Tumour tissue from solid tumours was minced to pieces 1 mm^3 in size and tumour cells were then isolated using collagenase dispersion and Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation as described previously [10]. Leukaemic cells were obtained from bone marrow or peripheral blood by 1077 g/ml Ficoll–Paque (Pharmacia) density gradient centrifugation [10, 11]. Viability was determined by the trypan blue exclusion test and the proportion of tumour cells was judged by inspection of May-Grünwald-Giemsa-stained cytocentrifugate preparations by a cytopathologist. Culture medium RPMI-1640 (Hyclone, Cramlington, U.K.) supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin was used throughout. Cells were cryopreserved in medium containing 10% dimethylsulphoxide (DMSO; Sigmal Chemical Co., St. Louis, Missouri, U.S.A.) and 90% heat-inactivated FCS by initial freezing for 24 h in −70°C, followed by storage in liquid nitrogen. Both fresh and cryopreserved samples were used in this study. It has previously been shown that cryopreservation does not appear to affect cytotoxic drug sensitivity using the present technique [11, 12].

Table 1. Cytotoxic effects of 2.5 μg/ml Calcein/AM on primary cultures of tumour cells from different tumour types

Diagnosis (*n*)	Mean SI value (S.D.)	*In vitro* response rate (%)*
Acute lymphocytic leukaemia (27)	24.4 (17.9)	92
Acute myelocytic leukaemia (27)	17.3 (16.6)	92
Chronic lymphocytic leukaemia (11)	15.1 (15.2)	91
Chronic myelocytic leukaemia (7)	17.1 (8.9)	100
Non-Hodgkin's lymphoma (8)	10.2 (6.7)	100
Colon cancer (15)	84.7 (23.9)	7
Non-small cell lung cancer (21)	52.4 (35.8)	71
Ovarian cancer (14)	62.4 (29.2)	42
Sarcoma (6)	34.5 (19.7)	83
Breast cancer (4)	50.5 (13.2)	25
Solid paediatric tumours† (5)	34.6 (23.6)	80
Bladder cancer (4)	52.2 (16.7)	50
Assorted solid tumours‡ (14)	55.4 (38.1)	62
Total (163)	38.4 (32.2)	72

**In vitro* response rate was defined as the percentage of samples with a >50% decrease in survival index (SI). †Solid paediatric tumours include Wilms' tumour (3), Ewing's sarcoma (1) and neuroblastoma (1). ‡Assorted tumours include: small cell lung cancer (2), phaeochromocytoma (2), carcinoid (2), melanoma (1), choriocarcinoma (1), paraganglioma (1), cancer of the appendix (1), carcinoma of the parotide (1), carcinoma of the fallopian tube (1) and unknown primary (2).

Reagents and drugs

Fluorescein diacetate (FDA; Sigma) was dissolved in DMSO and kept frozen (−20°C) as a stock solution (10 mg/ml) protected from light. Calcein/AM was obtained from Molecular Probes (Eugene, Oregon, U.S.A.). Cytosine arabinoside (AraC), amsacrine (Amsa), vincristine (Vcr), cisplatin (Cisp), carboplatin (Carbop), daunorubicin (Dnr), mitoxantrone (Mit), vinorelbine (Vino) and doxorubicin (Dox) were obtained from commercial sources. 4-Hydroperoxycyclophosphamide (4HC), an active metabolite of cyclophosphamide, was a kind gift from Asta Pharma, Stockholm, Sweden. CdA (2-chlorodeoxyadenosine) was obtained from Dr Zygmunt Kasimiensuk, of the Foundation for Diagnostics and Therapy (Warsaw, Poland). Cyclosporin A (CyA) was a kind gift from Sandoz (Basel, Switzerland). Calcein/AM was generally tested at two or five different concentrations, whereas the drugs used for comparison were tested at empirically derived cut-off concentrations (EDCC) as previously described [10, 11]. In short, EDCC is defined as the concentration of an anticancer drug giving a significant between tumour sample variability in cell survival index (SI), thereby facilitating the assessment of relative *in vitro* activity. Experimental plates were prepared with 20 μl/well of drug solution at 10 times the desired final concentration with the aid of a programmable pipetting robot (PROPETTE; Perkin Elmer, Norwalk, Connecticut, U.S.A.). The plates were stored frozen at −70°C until further use. The experiments were performed with continuous drug exposure.

Measurement of cytotoxicity

The principal steps of the fluorometric microculture cytotoxicity assay (FMCA) have been described previously [10, 11]. On day one, 180 μl of the tumour cell preparation (0.5–1 × 10^5 and 2.5–5 × 10^5 cells/ml for solid and haematological samples, respectively) was seeded into the wells of V-shaped 96-well experimental microtitre plates (Nunc, Roskilde, Denmark) prepared as described above. Six blank wells received only culture medium and six wells with cells but without drugs served as controls. The culture plates were then incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. At the end of the 72 h incubation period, the plates were centrifuged (200*g*, 5 min) and the medium removed by aspiration in the microtitre plate washer (Dynatech Laboratories, Chantilly, Virginia, U.S.A.). After one wash with phosphate-buffered saline (PBS), 100 μl of PBS containing FDA (10 μg/ml) was added columnwise to control, experimental and blank wells with the aid of an automated 96-well dispenser, Multidrop (Labsystems OY, Helsinki, Finland). Subsequently, the plates were incubated for 1 h before the fluorescence (ex 480, em 530) was read in the Fluoroscan 2 (Fluoroscan 2; Labsystems). The fluorometer was blanked against wells containing PBS with the fluorescent dye but without cells. Quality criteria for a successful assay included a fluorescein signal in control cultures of >5 × mean blank values, coefficient of variation (CV) in control cultures of <30% and >70% of tumour cells prior to

incubation. The overall success rate using these criteria was approximatly 80%. Only successfully analysed samples are reported here. In separate experiments, it was verified that the influence of 2.5 μg/ml Calcein/AM on the FDA signal was generally less than 5% of control values. In addition, for wells containing Calcein/AM the differential staining cytotoxicity assay (DiSC) [12, 13] was used to verify the accuracy of the fluorescent readings.

Quantification of cytotoxicity assay results

The results obtained are presented as SI defined as fluorescence as a per cent use of control cultures, with blank values subtracted. A drug producing a SI of ≤50% was regarded as active in that sample. The *in vitro* response rate for a particular drug was defined as the proportion (%) of samples showing a SI of ≤50% for that drug.

Statistical analysis

SI values at different drug concentrations for the haematological and solid tumours were compared using the Students *t*-test for unpaired comparisons. The Chi square test was used for comparison of proportions. Pearsons' correlation coefficient was calculated to compare the activity of Calcein/AM with standard chemotherapeutic drugs. The effect of CyA was tested using Students *t*-test for paired observations [14].

RESULTS

A concentration-dependent Calcein/AM-induced decrease in SI was observed for both haematological and solid tumour types (Figure 1). The differences between the solid tumour group and the haematological tumours were statistically significant ($P < 0.01$–0.001) at the three highest concentrations tested. When the *in vitro* response rates, calculated as the fraction of samples showing a decrease in SI of ≥50% were determined, a similar pattern was apparent (Table 2). The difference in response rates between haematological and solid tumours was statistically significant for Calcein/AM at 0.5, 2.5 and 12.5 μg/ml ($P = 0.038$–< 0.001). Haematological tumours also showed a significantly higher *in vitro* response rate at EDCC for Dox, Vcr, Amsa and AraC ($P < 0.001$) but not for cisplatin ($P = 0.24$). The ratio of solid tumour *in vitro* response rates over those for haematological tumours (S/H ratio) increased in a manner that was concentration dependent. Although the S/H ratio for Calcein/AM at 2.5 μg/ml was lower than that for cisplatin, they were both significantly higher than those obtained for leukaemic-specific agents (AraC and Amsa), Vcr and Dox (Table 2).

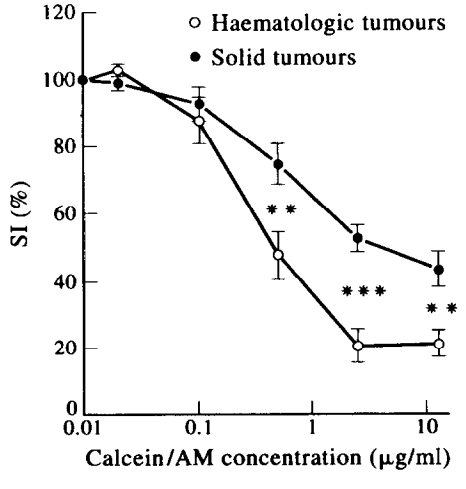

Figure 1. Effect of increasing concentrations of Calcein/AM on survival index (SI) in haematological (○; $n = 22$) and various solid tumours (●; $n = 15$). The results are presented as mean values ± SE. **$P < 0.01$; ***$P < 0.0001$.

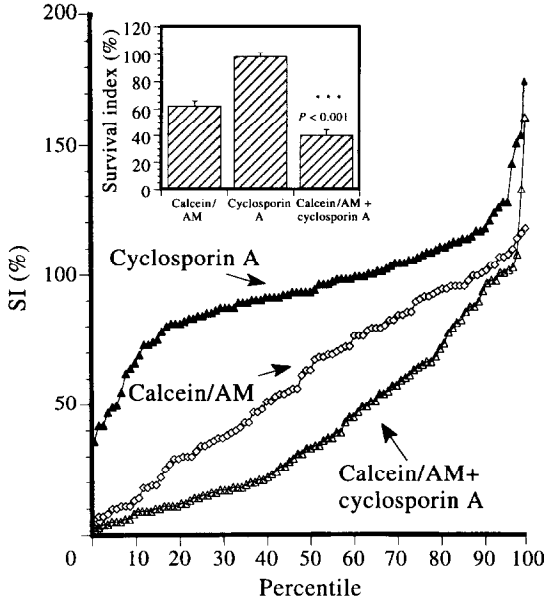

Figure 2. The probability distribution of survival index (SI) values for 0.5 μg/ml Calcein/AM alone (◇), 3 μg/ml cyclosporin A (▲) and their combination (Δ) for 98 tumour samples (60 haematological and 38 solid tumours). The inset shows the effect of cyclosporin A alone (3 μg/ml), Calcein/AM (0.5 μg/ml) and their combination on mean SI values ($n = 98$). ***$P < 0.001$.

At a concentration of 2.5 μg/ml Calcein/AM, low mean SI values (10.2–24.4%) and high *in vitro* response rates (91–100%) were observed for all haematological tumours tested (Table 1). In the solid tumour group, sarcomas and childhood tumours showed the lowest mean SI values (34.5 and 34.6%, respectively) and highest response rates (83% and 80%, respectively). Non-small cell lung cancer also showed a high *in vitro* response rate (71%) with a mean SI value of 52.4%. Colon cancer samples were largely insensitive to Calcein/AM as evidenced by a high mean SI (84.7%) and a response rate of 7%. The remaining solid tumour types showed intermediate mean SI values (50–55%) and response rates (25–62%).

Crossresistance analysis showed significant and expected correlations (> 0.70) for standard drugs belonging to the same mechanistic class (Table 3). For Calcein/AM, the highest correlation between SI values was observed with Dox ($r = 0.66$, $n = 142$) whereas the correlations to the other mechanistic classes of cytotoxic drugs were moderate ($r = 0.46$–0.59, $n = 106$–142) (Table 3). The correlation to paclitaxel, a promotor of microtubuli assembly, was also low ($r = 0.46$, $n = 143$, data not shown).

In Figure 2, the distribution of SI values in response to 0.5 μg/ml Calcein/AM was compared with those obtained with 3 μg/ml of CyA and the combination of these drugs for 98 samples (60 haematological and 38 solid tumour samples). Whereas the majority of samples were only marginally affected by CyA alone, the distribution curve for the combination of CyA + Calcein/AM was clearly shifted to the right from that

Table 2. In vitro *response rate (%)* and solid/haematological tumour ratios (S/H) of Calcein/AM and standard drugs*

Tumour type	Calcein/AM concentration					Dox	Cisp	Vcr	Amsa	AraC
	0.02 μg/ml	0.1 μg/ml	0.5 μg/ml	2.5 μg/ml	12.5 μg/ml	0.5 μg/ml	2.5 μg/ml	0.5 μg/ml	1 μg/ml	0.5 μg/ml
Haematological tumours	0/22 (0)	3/22 (14)	43/70 (61)	75/80 (94)	20/22 (91)	54/67 (81)	24/67 (36)	34/70 (49)	49/70 (70)	26/70 (37)
Solid tumours	0/15 (0)	0/15 (0)	10/54 (19)	42/81 (52)	8/13 (62)	20/76 (26)	20/75 (27)	6/67 (9)	2/38 (5)	0/38 (0)
P value†	—	0.14	<0.001	<0.001	0.038	<0.001	0.24	<0.001	<0.001	<0.001
Total	0/37 (0)	3/37 (8)	53/124 (43)	117/161 (73)	28/35 (80)	74/143 (52)	44/142 (31)	40/137 (29)	51/108 (47)	26/108 (24)
S/H ratio	—	<0.08	0.31	0.55	0.68	0.32	0.75	0.18	0.07	<0.03

Dox, doxorubicin; Cisp, cisplatin; Vcr, vincristine; Amsa, amsacrine; AraC, cytosine arabinoside; S/H ratio, solid tumour *in vitro* response rates over those for haematological tumours. *Number of samples showing >50% decrease in SI/total number of samples tested (%). †Comparison of *in vitro* response rates between haematological and solid tumours using the Chi square test.

Table 3. Correlations between Calcein/AM and standard agents with different proposed mechanisms of action

Drugs	Calcein/AM (2.5 μg/ml)	CdA (0.2 μg/ml)	Mit (0.5 μg/ml)	Carbop (25 μg/ml)	Dnr (0.5 μg/ml)	Vino (2.5 μg/ml)
Alkylating agent (4-HC, 2 μg/ml)	0.48	0.63	0.69	0.33	0.46	0.46
Antimetabolite (AraC, 0.5 μg/ml)	0.47	**0.77***	0.65	0.32	0.50	0.46
DNA-intercalator (Dox, 0.5 μg/ml)	0.66	0.63	**0.85**	0.53	**0.79**	0.37
Platinum compound (Cisp, 2.5 μg/ml)	0.47	0.44	0.51	**0.82**	0.55	0.60
Topo II inhibitor (Amsacrine, 1 μg/ml)	0.59	0.67	**0.82**	0.50	**0.84**	0.51
Tubulin interactive (Vcr, 0.5 μg/ml)	0.46	0.39	0.51	0.33	0.64	**0.80**

CdA, 2-chlorodeoxyadenosine; Mit, mitoxantrone; Carbop, carboplatin; Dnr, daunorubicin; Vino, vinorelbine; 4-HC, 4-hydroperoxycyclophosphamide; AraC, cytosine arabinoside; Dox, doxorubicin; Cisp, cisplatin; Topo II, topoisomerase II; Vcr, vincristine.
*Correlation coefficients >0.70 are underlined and in bold.

of Calcein/AM alone, indicating CyA-induced potentiation. The effect of CyA on Calcein/AM-induced cytotoxicity was statistically significant (Figure 2, inset).

DISCUSSION

In the present paper, significant cytotoxic activity for Calcein/AM on primary cultures of tumour cells from both haematological and solid tumours has been demonstrated at relatively low concentrations. Although the drug was more active against haematological tumours, substantial activity was observed against solid tumours. The relative activity of Calcein/AM at 2.5 μg/ml (and concentrations 5 times higher and lower) against solid tumours, as characterised by the S/H ratio, was more pronounced not only compared with EDCCs of the leukaemic-specific agents AraC and Amsa, but also with Dox with clinically documented solid tumour activity. From a methodological point of view, it should be recognised that EDCC is derived purely from *in vitro* activity data aimed at optimising the conditions for differentiation between sensitive and resistant tumour cell samples. Thus, EDCC does not necessarily reflect, for example, clinically achievable concentrations. However, it should be noted that the EDCCs of Dox and Cisp are very similar to reported peak plasma concentrations [15]. The robustness of the comparisons is also suggested by the relatively large concentration interval (0.5–12.5 μg/ml) of Calcein/AM with equal or higher S/H ratios compared to Dox at EDCC. Similar S/H ratios for the standard drugs were also observed when using IC_{50}s from equal numbers (20 haematological and 30 solid tumour samples) of consecutive non-paired samples (not shown). Nevertheless, due to the general toxicity profile of Calcein/AM, which is unknown at present, it obviously cannot be inferred that relevant *in vivo* antitumour activity will be achievable at tolerable concentrations.

The mechanism for calcein-induced cell death is not evident although the delayed loss of membrane integrity that has been previously reported, a characteristic feature of apoptosis, suggests an active process [8]. The cellular target for calcein is also not clear, but the cytotoxic effect requires trapping of calcein intracellularly [8]. Crossresistance analysis revealed moderately high correlations between Calcein/AM and Dox, a drug known to be part of the MDR crossresistance phenotype. Furthermore, in many samples, the effect of Calcein/AM was potentiated by the Pgp-blocker CyA. This is compatible with previous observations in established cell lines demonstrating that the AM-ester of calcein is a substrate for Pgp [5–7]. Although the utility of an anticancer agent may be hampered by the sensitivity to Pgp, resistance modifiers have been shown to increase significantly calcein accumulation and potentiate cytotoxicity [6, 8]. Furthermore, the relevance of Pgp for drug

resistance in the clinical setting is still uncertain. As the drug is reported to have a predominant cytoplasmic distribution [1, 2], direct interactions with intranuclear molecules seem less likely but cannot be completely excluded. Tubulin is one potential cytoplasmic target molecule for anticancer drugs, but the correlation with Vcr and paclitaxel was low. The relatively low correlation with Vcr compared to Dox suggests that Calcein/AM may share other mechanisms of Dox action that determine the spectrum of activity. Apart from being a topoisomerase II inhibitor, Dox has also been shown to affect other cellular functions, including the generation of free radicals, alterations in plasma membrane function and inhibition of activity in intracellular signal systems [16]. However, definitive evidence on mechanism of action is lacking and the intracellular target structures for the calcein molecule remain to be determined.

The possibility of *in vivo* application of the principle of cytoplasmic targeting of Calcein/AM and related compounds by esterase-dependent trapping has not been evaluated. Cellular drug delivery by esterase-dependent trapping may theoretically provide some protection for the haematological precursor cell progeny that show low non-specific esterase expression [17]. Some preferential cellular uptake in solid tumours may also be accomplished as, at least on a per cell basis, many solid tumours show considerably higher esterase activity compared with their haematological counterparts [10]. It should be noted, however, that not only the intracellular concentration of calcein, but also other factors, such as target expression and/or sensitivity, appear to influence the sensitivity of a given cell type to Calcein/AM [8]. The cytotoxic effect of calcein on sensitive cell lines shows no apparent schedule dependency *in vitro* [8], which may be a potential pharmacokinetic advantage when Calcein/AM is evaluated *in vivo*. This would allow rapid distribution and cellular retention from a single bolus administration. There may be several obstacles, however, to the passage of AM-esters between the intravenous compartment and the tumour cell cytoplasm. The extent of AM-ester crossing of physiological barriers and the impact of membrane-bound Pgp and/or other transport proteins regulating AM-ester distribution are clearly potential pitfalls. Another potentially important limiting factor for *in vivo* application may be the extent to which de-esterification occurs in extracellular compartments. Plasma esterase activity may rapidly inactivate Calcein/AM by removing the ester groups before any tissue distribution has taken place [18]. This problem may be even more pronounced using murine species as models due to their high endogenous plasma esterase activity [18]. However, a recent study using AM-esters of a calcium chelator demonstrated the feasibility of delivering this AM-ester into the cytoplasm of central neurons *in vivo* using intravenous administration in rats [19]. This was also the case for other AM-esters, including those with structural similarity to Calcein/AM [19]. Thus, the *in vitro* testing of calcein/AM in murine tumour models may indeed be a feasible task and such studies are currently underway.

In conclusion, we have demonstrated that Calcein/AM shows substantial cytotoxic activity at relatively low concentrations in both haematological and solid tumour cell samples from patients. The cellular target appears to be located in an intracellular compartment to which the AM-ester is delivered by esterase-dependent trapping. These observations warrant further preclinical investigation of the antitumour activity of Calcein/AM and related compounds.

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