Rapid up-regulation of cyclooxygenase-2 by 5-fluorouracil in human solid tumors

Stuart J. Mercer[a], Federica Di Nicolantonio[a], Louise A. Knight[a], Francis G. Gabriel[a], Pauline A. Whitehouse[a], Sanjay Sharma[a], Augusta Fernando[a], Pradeep Bhandari[b], Shaw S. Somers[b], Simon K. Toh[b] and Ian A. Cree[b] for the NHS Collaborative Research Programme on Predictive Oncology

Inhibition of cyclooxygenase (COX)-2 has been associated with reduced growth of malignant cells. Current therapy of gastrointestinal carcinomas involves the use of 5-fluorouracil (5-FU)-based chemotherapy and we have therefore studied the effect of this agent on the expression of COX-2. COX-2 expression was measured by quantitative RT-PCR in biopsies from a series of 14 esophageal carcinomas, six of which had paired samples taken before and after chemotherapy, and in tumor-derived cells exposed to 5-FU *in vitro* from a series of 44 tumors, including breast, ovarian, esophageal and colonic carcinomas. COX-2 expression was increased by exposure to 5-FU or 5-FU combination chemotherapy in all the tumor types studied, whether measured in biopsies taken before and after 5-FU-based chemotherapy (4-fold increase, $p<0.015$) or in primary cells exposed to drugs *in vitro* (24-fold increase, $p<0.001$). A modest increase of COX-2 mRNA was also seen after *in vitro* treatment of cells with cisplatin. In contrast, doxorubicin and paclitaxel caused no up-regulation *in vitro*, while irinotecan caused inhibition of COX-2 (2.7-fold decrease, $p<0.01$). These data provide a molecular rationale for clinical trials of combination chemotherapy with COX-2 inhibitors. ***Anti-Cancer Drugs*** **16:495–500 © 2005 Lippincott Williams & Wilkins.**

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[a]Translational Oncology Research Centre, Department of Histopathology and [b]Solent Centre for Digestive Diseases, Queen Alexandra Hospital, Portsmouth, UK.

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The first two authors contributed equally to this work.

Correspondence to I. A. Cree, Translational Oncology Research Centre, Michael Darmady Laboratory, Department of Histopathology, E Level, Queen Alexandra Hospital, Portsmouth PO6 3LY, UK.
Tel: +44 23 9228 6378; fax: +44 23 9228 6379;
E-mail: ian.cree@port.ac.uk

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Introduction

Cyclooxygenase (COX)-2 is involved in the synthesis of prostaglandins that are known to enhance cell proliferation and growth in both normal and tumor cells [1]. COX-2 is expressed in the vast majority of carcinomas, including breast, ovarian and gastrointestinal cancer [2]. It plays an important role in carcinogenesis (through effects on proliferating cells) and in prognosis (by increasing the metastatic potential of tumor cells). COX-2 expression has been implicated in the pathogenesis of gastrointestinal cancer [3] and has been found to be associated with poor survival of patients with these tumors, making COX-2 as an attractive target for both cancer prevention and treatment [4]. Indeed, there is increasing evidence that non-steroidal anti-inflammatory drugs and more selective COX-2 inhibitors might play a role in both chemoprevention and treatment of several cancer types [5,6]. COX-2 expression is known to be induced by a variety of factors, including hormones, pro-inflammatory cytokines, growth factors and tumor promoters. However, there are no reported studies investigating the effects of chemotherapeutic agents on the expression of COX-2.

Materials and methods

In this study we examined COX-2 expression in 20 esophago-gastric biopsies taken before and after chemotherapy, 12 of which were paired samples from the same six patients. Expression of COX-2 relative to housekeeping genes was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). We included up to four housekeeping genes that did not show significant changes following exposure of cells to cytotoxic agents to ensure accurate comparison of results from different samples.

Tissue samples

Of the 44 solid tumor samples studied in short-term cell culture, seven were esophago-gastric, 16 were breast, 11

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were ovarian carcinomas and 10 were colorectal tumors. Patients consisted of 33 females and 11 males, having a median age of 68 (range 37–94). Nine patients received previous chemotherapy [epirubicin + cisplatin + 5-fluorouracil (5-FU) (ECF) $n = 4$; mitoxantrone + paclitaxel $n = 1$; carboplatin + paclitaxel $n = 4$], while 35 of 44 had no previous treatment. For the *in vivo* studies, material was obtained from 14 esophageal cancer patients (13 M:1 F, median age 64, range 42–78) at diagnosis. For six of these patients, further tumor tissue was also available after treatment with 2 cycles of ECF combination chemotherapy. All tumor samples were removed as part of patient treatment, with consent for tissue donation and local research ethics committee approval for use of the tissue surplus to diagnostic requirements for cellular and molecular assays.

Drugs

Cisplatin, epirubicin, 5-FU and irinotecan were obtained from the pharmacy at Queen Alexandra Hospital. Cisplatin and 5-FU were stored at room temperature, while epirubicin and irinotecan were stored at –20°C. The drug concentrations (TDC) to which tumor derived cells were exposed were as follows: 5.0 μM for cisplatin, 170 μM for 5-FU, 0.431 μM for epirubicin and 74 μM for irinotecan. The ECF combination was made up by adding the three drugs concurrently at the beginning of the ATP cell viability assay and diluted in a constant ratio; sequential studies were not performed.

Short-term cell culture

Tumor tissue or fluid was taken by a histopathologist or surgeon under sterile conditions, and transported to the laboratory in cell culture medium of Dulbecco's modified Eagle's medium (DMEM; Sigma, Poole, Dorset, UK; cat. no. D5671) with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma; cat. no. P0781) at 4°C. Cells were obtained from solid tumors by gentle enzymatic dissociation, usually 0.75 mg/ml collagenase (Sigma; cat. no. C-8051) overnight. Viable tumor-derived cells were separated from dead cells and debris by density centrifugation (Histopaque 1077-1; Sigma), washed, counted and resuspended to 200 000 cells/ml. In the meantime, 96-well polypropylene microplates (Corning-Costar, High Wycombe, UK; cat. no. 3790) were prepared with each drug/combination, according to the protocol set by Andreotti *et al.* [7]. Approximately 20 000 cells/well were added to the plates to a final volume of 200 μl/well. The plates were then incubated at 37°C in 5% CO_2 for 6 days, after which the degree of cell inhibition was assessed by measurement of the remaining ATP in comparison with negative control (no drug, M0) and positive control (maximum inhibitor, MI) rows of 12 wells each. Prior to cells lysis with an ATP-extracting reagent, 150 μl of cell suspension was removed from each well, centrifuged, washed with phosphate-buffered saline (PBS) and stored at –80°C in a GTIC-containing solution (lysis buffer RA1; Macherey-Nagel, Düren, Germany; cat. no. 740961) until further molecular analysis. ATP was extracted from the remaining 50 μl cell suspension and measured by light output in a microplate luminometer (Berthold Diagnostic Systems, Pforzheim, Germany) following addition of luciferin–luciferase.

RNA extraction

Cells obtained after enzymatic dissociation from endoscopic esophageal biopsies were either resuspended in RNAlater (Ambion, Huntingdon, UK) or lysed with buffer RA1 and stored at –80°C until RNA extraction. For the *ex vivo* studies, cells that survived after drug exposure in the ATP-TCA were pooled from at least six wells to increase nucleic acid yield and stored as described above. Total RNA was extracted from at least 50 000 cells with a commercially available kit (NucleoSpin RNA II Mini; Macherey-Nagel; cat. no. 740955) according to the manufacturer's instructions. The protocol included a DNase digestion step to prevent carry-over of genomic DNA in further analysis.

qRT-PCR

A two-step protocol was employed. First, total RNA was reverse transcribed by using the Promega reverse transcription system (Promega, Southampton, UK; cat. no. A3500) including 8 μl RNA, 0.5 μg random primers, 20 U of recombinant RNasin ribonuclease inhibitor and 15 U of reverse transcriptase AMV (Promega; cat. no. M9004) to each 20-μl reaction. The resulting cDNA was amplified by real-time quantitative PCR on a Bio-Rad iCycler instrument (Bio-Rad, Hemel Hampstead, UK). The constituents of each PCR reaction (25 μl) were 1 μl of cDNA (or H_2O), 200–500 nM of each primer (Table 1), 200 μM each dATP, dCTP and dGTP, 400 μM dUTP, 3.0–5.0 mM $MgCl_2$, 0.125 U AMPErase UNG, 0.625 U of AmpliTaq Gold DNA polymerase and 1 × SYBR Green PCR buffer (all reagents were from Applied Biosystems, Warrington, UK). Product amplification was performed up to 45 PCR cycles, after uracil removal (2 min at 50°C) and polymerase activation (10 min at 95°C). Each two-step PCR cycle comprised denaturing (15 s at 95°C), annealing and extending (1 min at 60°C). At the end of each run a final melt curve cycle (cooling to 50°C and then increasing stepwise 1–95°C) was performed to exclude the presence of primer-dimer artifacts. All products were of expected size on agarose gel electrophoresis (Table 1) and specificity was checked using internal Taqman probes (Applied Biosystems).

We have used at least three housekeeping genes for each experiment chosen among the following: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), human porphobilinogen deaminase (PBGD) and TATA box binding protein (TBP). The internal reference genes were selected due to their relative low abundance in normal

Table 1 Sequence of primers (forward and reverse) used for qPCR experiments

Name	Sequence 5′–3′ (forward and reverse primer)	Product length	Reference
GAPDH (NM002046)	GAA GGT GAA GGT CGG AGT C	226	
	GAA GAT GGT GAT GGG ATT TC		
HPRT1 (NM000194)	TCA GGC AGT ATA ATC CAA AGA TGG T	84	[8]
	AGT CTG GCT TAT ATC CAA CAC TTC G		
PBGD (NM000190)	CTG CAC GAT CCC GAG ACT CT	98	[9]
	GCT GTA TGC ACG GCT ACT GG		
TBP (X54993)	CAC GAA CCA CGG CAC TGA TT	89	[10]
	TTT TCT TGC TGC CAG TCT GGA C		
COX-2 (M90100)	CCT TCC TCC TGT GCC TGA TG	81	[11]
	ACA ATC TCA TTT GAA TCA GGA AGC T		

GenBank accession numbers for each gene are indicated in brackets. When no reference is indicated, the primers were designed using an old version of the software Primer 3.0, available at the following website: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/.

tissue [12]. The housekeeping genes were amplified parallel to the target genes in separate vessels. When possible, primer sequences (listed in Table 1) were chosen to span exon boundaries. A positive control (pooled cDNA from a variety of human tumors, including breast, ovarian, colorectal and esophageal carcinoma) and negative controls with no template and RT-negative as template were added in every experiment. All assays were run in triplicate. Validation experiments were run to show that the efficiencies of the target and reference genes amplifications were approximately equal; the efficiencies of GAPDH, HPRT1, PBGD, TBP and COX-2 reactions were 99, 98, 101, 98 and 97, respectively. The PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, C_t; 10 standard deviations above the mean fluorescence generated during the baseline cycles) was determined, and a comparative C_t method was then used to measure relative gene expression [13]. The following formula was used to calculate the relative amount of the transcript in the sample: $2^{-\Delta\Delta C_t}$, where ΔC_t is the difference in C_t between COX-2 and the mean of the at least two reference genes and $\Delta\Delta C_t = \Delta C_t$ of non-drug exposed cells $- \Delta C_t$ of drug-exposed cells, for the *ex vivo* experiments, or $\Delta\Delta C_t = \Delta C_t$ of the pre-chemotherapy sample $- \Delta C_t$ of the post-chemotherapy sample, for matched tumor biopsies.

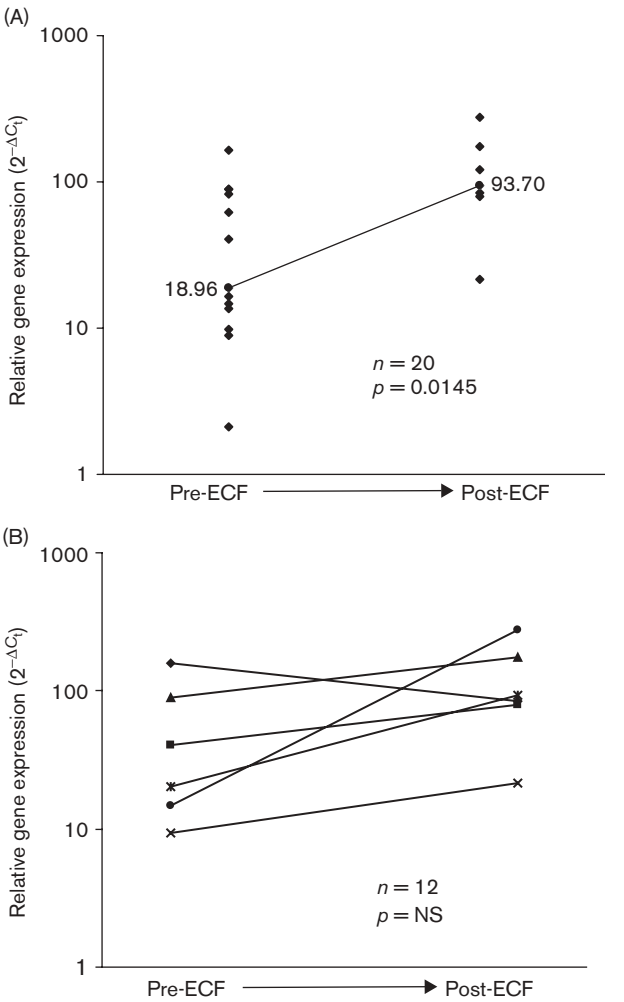
Results

Figure 1(A) shows the effect of 2 cycles of combination (ECF) chemotherapy on COX-2 expression in unpaired esophageal biopsies; the expression of COX-2 in post-chemotherapy biopsies was at least 4-fold higher than biopsies from the chemotherapy naïve group ($p = 0.0145$; Mann–Whitney U-test). When a small subset of paired biopsies from six patients were analyzed, a trend towards increased expression of COX-2 was also observed (Fig. 1B), with some variation between patients.

These results raised the possibility that COX-2 levels in neoplastic cells might be affected by chemotherapy and we therefore exposed tumor-derived cells to individual chemotherapeutic agents *in vitro* to establish which drugs exhibited this phenomenon.

To determine whether these effects might occur rapidly after the start of chemotherapy, we treated tumor-derived

Fig. 1

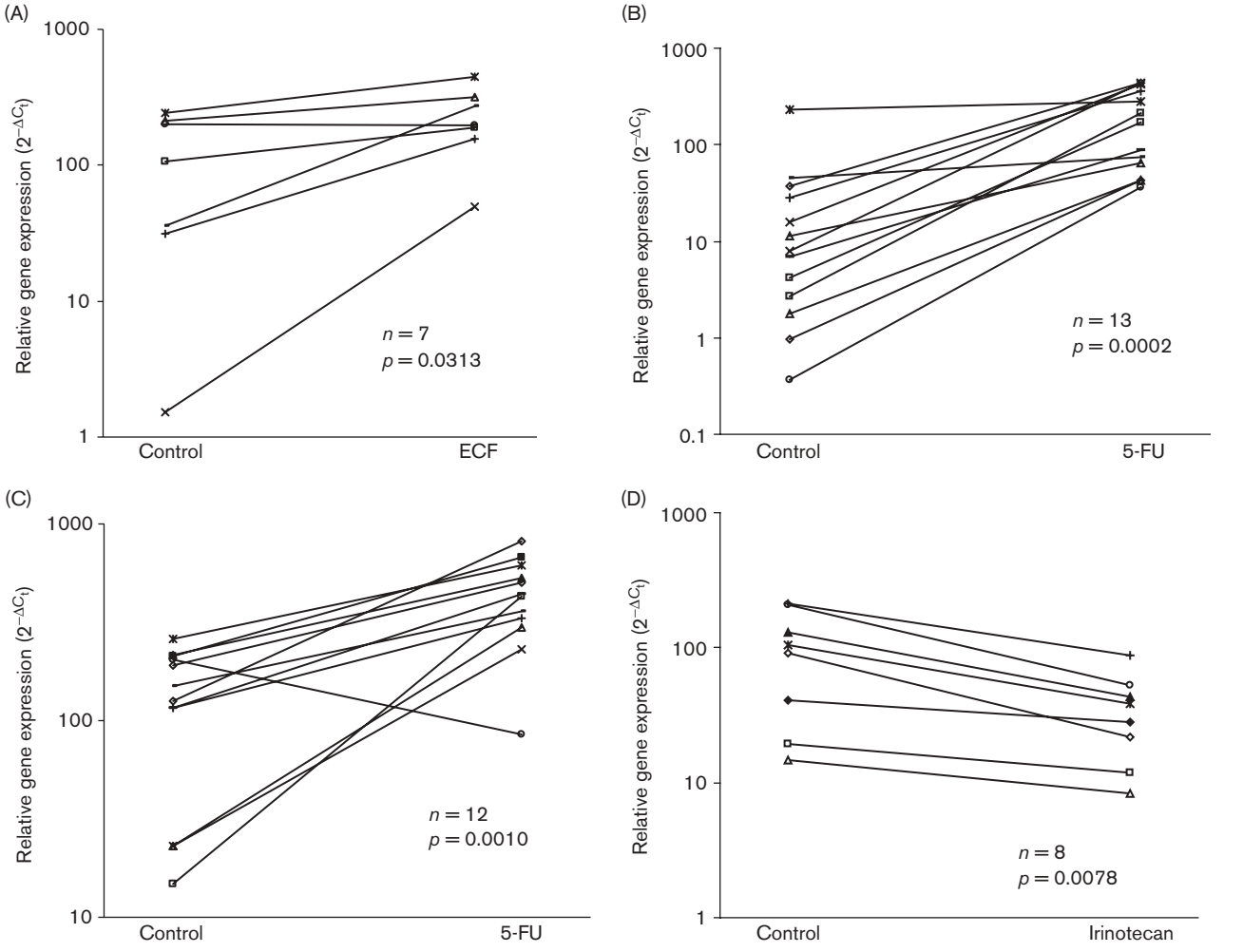


Relative expression of COX-2 in esophageal tumor biopsies given on a logarithmic scale: $2^{-\Delta Ct}$, where ΔC_t is the difference in threshold cycle (C_t) between COX-2 and the mean of at least two housekeeping genes. Each dot represents an individual sample, while the line indicates the median values. (A) The expression of COX-2 shows a significant increase in patients following therapy (unpaired data). (B) The expression of COX-2 increased in five of six patients (paired samples) after ECF chemotherapy.

cells from seven esophageal, 10 colorectal, 11 ovarian and 16 breast carcinomas with various cytotoxic drugs in short-term cell culture. For these experiments, cells were obtained from solid tumors by gentle enzymatic dissociation and cultured for 6 days in a selective medium (Complete Assay Medium; DCS, Hamburg, Germany), as previously reported [14]. As before, COX-2 expression was measured by qRT-PCR. Patients consisted of 33 females and 11 males, with a median age of 68 (range 37–94): 35 of 44 had received no previous treatment. All tumor samples were removed as part of patient treatment, with consent for tissue donation and local research ethics committee approval for use of the material surplus to diagnostic requirements.

In general, we observed heterogeneity of COX-2 expression among different samples following *in vitro* exposure to cytotoxics. As shown in Fig. 2(A), the combination ECF increased COX-2 levels by a median value of 1.9-fold (range 1.0–32.5; $p = 0.0313$, Wilcoxon matched-pairs test) in esophageal tumor-derived cells. Notably, 5-FU on its own caused a median 24-fold (range 1.2–98.1; $p = 0.0002$, Wilcoxon) and 3-fold (range 0.4–28.8; $p = 0.0010$, Wilcoxon) up-regulation of COX-2 mRNA in breast (Fig. 2B) and gastrointestinal (Fig. 2C) carcinoma cells, respectively. A modest 1.8-fold (range 0.9–6.7; $p = 0.0005$, Wilcoxon) up-regulation of COX-2 mRNA was also seen in breast tumor-derived cells exposed to cisplatin, but not in ovarian tumor-derived cells (data not shown). Among the other cytotoxics tested, paclitaxel

Fig. 2



Relative expression of COX-2 in tumor-derived cells exposed to different cytotoxics. The results given on a logarithmic scale: $2^{-\Delta Ct}$, where ΔC_t is the difference in threshold cycle (C_t) between COX-2 and the mean of at least two housekeeping genes. Each dot represents an individual sample. *In vitro* treatment with ECF causes an up-regulation of COX-2 in upper gastrointestinal tumor-derived cells (A). The expression of COX-2 also shows a significant increase in breast (B) and gastrointestinal (C) tumor-derived cells following *in vitro* exposure to 5-FU. (D) The expression of COX-2 is decreased after *in vitro* treatment of colorectal tumor-derived cells with irinotecan.

and doxorubicin had no significant overall effect on COX-2 expression in either breast or ovarian tumor-derived cells (data not shown). Irinotecan, a topoisomerase I inhibitor, tended to decrease COX-2 in the gastrointestinal tumors cells (Fig. 2D): we observed a down-regulation of COX-2 mRNA in all eight gastrointestinal samples analyzed, with a median 2.7-fold decrease (range 1.5–4.2; $p = 0.0078$, Wilcoxon).

As we have used small biopsies and tumor-derived cells, the amount of material present did not allow us to measure COX-2 protein expression by Western blot or prostaglandin E_2 production: this should be attempted in larger studies. However, immunohistochemistry has been performed on a small number of samples obtained from patients ($n = 5$) before and after ECF chemotherapy, and we have noted up-regulation of COX-2 protein in three of five paired esophageal biopsies.

Discussion

The only existing data comparable with this study are from cell line studies. Evidence exists that COX-2 mRNA in breast cells can be induced by microtubule-interfering agents [15] and docetaxel, in particular [16]. In our study exposure to paclitaxel *in vitro* induced a 2-fold or more up-regulation of COX-2 in only five or 16 breast and two of eight ovarian samples tested. However, it must be noted that both studies from Subbaramaiah *et al.* [15,16] have only used one breast cell line, the 184B5/HER, and therefore their findings cannot be generalized. Recently, the same group has also showed that SN-38, a metabolite of irinotecan which we have found less active than irinotecan in our tumor-derived cells [14], was able to inhibit phorbol ester-mediated induction of COX-2 in human gastrointestinal cancer cell lines [17]. Although the authors did not report a direct effect of camptothecin derivatives on constitutive COX-2 levels, they suggested a link between topoisomerase I and COX-2. On the other hand, other studies suggest that the up-regulation of COX-2 by 5-FU may be part of a general anti-apoptotic response. Sun *et al.* [18] have demonstrated that forced COX-2 expression attenuated induction of apoptosis by 5-FU predominantly through inhibition of the cytochrome *c*-dependent apoptotic pathway; Grösch *et al.* [19] have also shown that the selective COX-2 inhibitor celecoxib is able to induce cell cycle arrest and apoptosis in cultured colon cancer cells. It must be highlighted, however, that these studies all used cell lines, and that both in the case of Sun *et al.* [18] and Yamaguchi *et al.* [17], the authors stimulated or forced the expression of COX-2.

The marked change in COX-2 expression following exposure to 5-FU may represent part of a re-growth phenomenon or give the cells a survival advantage. Up-regulation of COX-2 may therefore render cancer cells more resistant to 5-FU chemotherapy, in keeping with some previous reports that the combination of COX-2 inhibitors with fluoropyrimidines might be useful. Further studies are needed on this point, and it would valuable to build measurements of COX-2 into clinical trials involving 5-FU. Our data also provide a molecular rationale for the observed efficacy of combinations of topoisomerase I inhibitors with 5-FU in gastrointestinal cancers.

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