Differential regulation of cyclin D1 and cell death by bile acids in primary rat hepatocytes

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1Centro de Patogénesis Molecular, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal; and Departments of 2Medicine and 3Genetics, Cell Biology, and Development, University of Minnesota Medical School, Minneapolis, Minnesota

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Castro RE, Amaral JD, Solá S, Kren BT, Steer CJ, Rodrigues CM. Differential regulation of cyclin D1 and cell death by bile acids in primary rat hepatocytes. Am J Physiol Gastrointest Liver Physiol 293: G327–G334, 2007. First published April 12, 2007; doi:10.1152/ajpgi.00093.2007.—Ursodeoxycholic (UDCA) and tauroursodeoxycholic (TUDCA) acids modulate apoptosis and regulate cell-cycle effectors, including cyclin D1. In contrast, deoxycholic acid (DCA) induces cell death and cyclin D1. In this study, we explored the role of cyclin D1 in DCA-induced toxicity and further elucidated the antiapoptotic function of UDCA and TUDCA in primary rat hepatocytes. Cells were incubated with DCA and with or without UDCA or TUDCA for 8–30 h. In addition, hepatocytes were transfected with either an adenovirus expressing cyclin D1 or with a cyclin D1 reporter plasmid with or without bile acids. Finally, cells were cotransfected with short interfering RNA targeting p53. Unlike DCA, both UDCA and TUDCA reduced cyclin D1 expression and transcriptional activation, confirming our previous DNA microarray data. Furthermore, UDCA and TUDCA prevented DCA-induced cyclin D1 and cell death. Cyclin D1 overexpression increased DCA-induced Bax translocation, cytochrome c release, and apoptosis. However, UDCA and TUDCA were less efficient at decreasing cyclin D1 levels as well as DCA-induced changes with overexpression. Finally, after p53 silencing, the effects of cyclin D1 overexpression were almost completely abrogated, whereas UDCA and TUDCA cytoprotective potential was reestablished. In conclusion, cyclin D1 is a relevant player in modulating apoptosis by bile acids, in part through a p53-dependent mechanism.

apoptosis; Bax; liver; p53

UDRSEOXYCHOLIC ACID (UDCA) stimulates hepatobiliary secretion and inhibits liver cell apoptosis (24). We and others (3, 28, 30) have shown that UDCA and tauroursodeoxycholic acid (TUDCA) reduce the apoptotic threshold in several cell types through modulation of classical mitochondrial pathways.

The mechanisms by which UDCA and TUDCA target mitochondria are now being unfolded. UDCA has been shown to inhibit hydrophobic deoxycholic acid (DCA)-induced Bax translocation both in vivo and in vitro (29, 30). In addition, we have previously shown (36) that UDCA can specifically modulate the E2F-1/p53/Bax apoptotic pathway, abrogating E2F-1-induced p53 and p53-associated Bax expression. Furthermore, UDCA’s antiapoptotic effects involve the activation of nuclear steroid receptors coupled with the inhibition of the E2F-1/p53/Bax pathway (35). More recently, we have shown that UDCA and TUDCA regulate gene transcription, specifically apoptosis-, cell cycle- and proliferation-related genes (4). Cyclin D1 was found to be significantly downregulated by both bile acids. Interestingly, UDCA has previously been suggested to have a chemopreventive role in a rodent model of colonic carcinogenesis (10) and to protect against the development of human colon cancer (38). Other studies (40) have demonstrated that cyclin D1 may be involved in these processes.

Cyclin D1 is well known for regulating the G1 phase of the cell cycle by binding to and stimulating the activities of Cdk4 or Cdk6 (13). This process leads to phosphorylation and inhibition of the retinoblastoma protein, which no longer binds to and inhibits members of the E2F transcription factor family. Active E2F-1 can transactivate the expression of genes that are involved in the S-phase progression of the cell cycle and subsequent DNA synthesis (13). Disruptions in the formation of the Cdk-cyclin complexes may exacerbate cell growth and proliferation or, inversely, cell death. In this regard, several tumor types display amplification and/or overexpression of the cyclin D1 gene (15). Interestingly, elevated cyclin D1 levels can also lead to growth suppression and apoptosis (12, 33). Studies in human breast cancer cell lines have shown that increased expression of cyclin D1 initiates apoptotic events (26). Interestingly, p53 was found to be essential for cyclin D1 overexpression-induced Bax activation. However, other studies showed that both wild-type and mutant p53-expressing cells were sensitive to induction of apoptosis by overexpression of cyclin D1 (12). Thus it appears that cyclin D1-induced apoptosis may occur through both p53-dependent and -independent mechanisms.

We have previously shown (4) that UDCA and TUDCA modulate cell cycle- and apoptosis-related genes in primary rat hepatocytes, and we identified cyclin D1 as a main target. Here we further explored the role of cyclin D1 in DCA-induced toxicity as well as UDCA and TUDCA antiapoptotic function in hepatocytes. Our results indicate that DCA-induced apoptosis is associated with cyclin D1-dependent Bax translocation through nontranscriptional p53-dependent mechanisms. In addition, inhibition of cyclin D1 by UDCA and TUDCA attenuated DCA-induced effects.

MATERIALS AND METHODS

Hepatocyte cultures and bile-acid treatment. Rat primary hepatocytes were isolated from male Sprague-Dawley rats (100–150 g) by collagenase perfusion (19). After isolation, hepatocytes were resus-
pended in Williams’ E medium (Sigma-Aldrich, St. Louis, MO) with supplements and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) (36). Cells were plated at either 5 × 10^5 cells/cm^2 for viability assays and apoptosis detection or at 2.5 × 10^5 cells/cm^2 for other experiments. Twenty-four hours after being plated, cells were treated with either 100 μM UDCA or TUDCA (Sigma-Aldrich) or no addition (control) for 1 h. Cells were then exposed to 50 μM DCA (Sigma-Aldrich) for an additional 8, 18, or 30 h. Bile-acid concentrations were selected based on previous cell-culture and animal studies and are the same order of magnitude as those found in patients with cholestatic liver disease undergoing UDCA therapy (24).

**Measurement of cell death.** Cell viability was measured by lactate dehydrogenase (LDH) viability assays (Sigma-Aldrich) using culture medium. In addition, Hoechst labeling of attached cells was used to detect apoptotic nuclei (36). Three random microscopic fields per sample of ~150 nuclei were counted, and mean values were expressed as the percentage of apoptotic nuclei. The terminal transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assays were performed according to the manufacturer’s recommendations (Sigma-Aldrich, St. Louis, MO).

**Cyclin D1 transfections and luciferase assays.** Transfections were performed by using a cyclin D1 adenovirus expression construct and a cyclin D1 promoter-driven luciferase reporter. The recombinant cyclin D1 adenovirus (ADV-D1) was constructed by ligating the EcoRI/HindIII fragment of human cyclin D1 into pACCMV.pLpA (1). An identical construct containing the nuclear-localizing variant of β-galactosidase (ADV-βgal) was used as control. The cyclin D1 promoter-driven luciferase construct consisted of a 1,745-bp human cyclin D1 promoter fused to the luciferase gene (25). Cells were infected with ADV-D1 or ADV-βgal 4 h after being plated at a dose of 20 plaque-forming units/cell. The medium containing the adenovirus was removed and replaced with virus-free medium after 2 h. Cells were treated with bile acids and were harvested for total, cytosolic, mitochondrial, and nuclear protein extraction and immunoblotting. Attached cells were also fixed for morphological detection of apoptosis.

In parallel experiments, 12 h after being plated, hepatocytes were transfected with 4 μg of cyclin D1 promoter-driven luciferase reporter by using conjugated polyethyleneamine (16). For normalization, cells were cotransfected with the chloramphenicol acetyltransferase (CAT) reporter construct, pCAT3-Control (Promega, Madison, WI). Transfection efficiencies of ~70% were determined by using a reporter plasmid expressing β-galactosidase and did not differ between reporter and expression plasmids (data not shown). Cells were treated with bile acids and were maintained in culture for additional 8, 18, or 30 h, after which they were harvested for CAT ELISA (Roche Applied Science, Indianapolis, IN) and luciferase assays (Promega).

**Short interference-mediated silencing of the p53 gene.** A pool of three target-specific, 20- to 25-nt short interfering RNAs (siRNAs) was used to knock down p53 gene expression in rats (Santa Cruz Biotechnology, Santa Cruz, CA). Control siRNAs containing a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA were used as control. Four hours after being plated, hepatocytes were transfected by using JetSI for siRNA (Polyplus Transfections, Illkirch, France). Four hours later, cells were transfected with ADV-D1 and were treated with bile acids. Floating and attached cells were harvested for total, cytosolic, and mitochondrial protein extraction followed by immunoblotting. Attached cells were fixed for Hoechst staining, and culture medium was collected for LDH assays.

**Immunoblotting.** Cellular distributions of Bax and cytochrome c were determined by using mitochondrial and cytosolic protein extracts as previously described (5). Forty micrograms of mitochondrial and cytosolic proteins were separated by 15% SDS-PAGE. Blots were incubated with a primary monoclonal antibody to either Bax (Santa Cruz Biotechnology) or cytochrome c (PharMingen, San Diego, CA). Mitochondrial contamination of the cytosolic protein extracts was determined by Western blot analysis of cytochrome c oxidase (subunit II). In addition, 10–50 μg of total protein extracts were separated on 10% SDS-PAGE, and blots were probed with either primary mouse monoclonal antibodies reactive with p53 and Bax or primary rabbit polyclonal antibodies to cyclin D1 (Santa Cruz Biotechnology). β-Acetin and cytochrome c oxidase (subunit II) were used as loading controls.

**Densitometry and statistical analysis.** The relative intensities of protein bands were analyzed by using the Quantity One version 4.6 densitometric analysis program (Bio-Rad Laboratories). Statistical analysis was performed by using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA) for the analysis of variance and Bonferroni’s multiple-comparison tests. Values of P < 0.05 were considered significant.

**RESULTS**

**UDCA and TUDCA reduce DCA-induced cell death and cyclin D1 expression.** In addition to its role in cell proliferation and oncogenesis, cyclin D1 is also involved in apoptosis (13). Furthermore, using microarray analysis, we (4) have previously identified cyclin D1 as a novel potential target of UDCA and TUDCA in primary rat hepatocytes. Therefore, we hypothesized that cyclin D1 may play a critical role in bile-acid-induced modulation of apoptosis and cell survival.

In this study, incubation of primary rat hepatocytes with DCA resulted in significant levels of general cell death (Fig. 1A) and apoptosis (P < 0.05, Fig. 1B). Annexin V/propidium iodide double staining further confirmed these results (data not shown). In contrast, UDCA and TUDCA inhibited DCA-induced cell death by ~50% (P < 0.05) throughout the time course (Fig. 1, A and B). Similar results were obtained when apoptosis was assessed by TUNEL staining (Fig. 1C).

Cyclin D1 protein increased above control after 18 h of incubation with DCA (P < 0.05; Fig. 2A). UDCA and TUDCA alone decreased cyclin D1 protein (P < 0.05; Fig. 2B) in agreement with reduced cyclin D1 mRNA transcripts in the microarray analysis. Importantly, UDCA and TUDCA abrogated DCA-induced cyclin D1 expression (P < 0.01 and P < 0.05, respectively). Semiquantitative RT-PCR showed a twofold increase in cyclin D1 gene by DCA (P < 0.05), which was inhibited by ~50% after preincubation with UDCA or TUDCA (data not shown). Furthermore, DCA also induced cyclin D1 promoter activity by ~2.5- and 3-fold at 8 and 18 h of incubation, respectively (P < 0.05; Fig. 2C). In agreement with the mRNA and protein-expression data, UDCA and TUDCA alone significantly decreased cyclin D1 promoter activity (P < 0.05) and DCA-mediated changes in cyclin D1 expression levels (P < 0.05).

**Cyclin D1 expression is involved in modulation of apoptosis.** On the basis of the information that bile acids differentially regulate cyclin D1 expression levels, we next investigated whether cyclin D1 contributed to modulation of apoptosis. ADV-D1 was transfected into cells, and immunoblot analysis showed that ADV-D1 transfection resulted in high-level expression of cyclin D1 (>20-fold) compared with cyclin D1 levels in ADV-βgal-infected cells (Fig. 3A). Freshly isolated primary hepatocytes are primarily in the G0 phase of the cell cycle. In the presence of growth factors, they slowly progress through G1 phase, entering S phase 42–45 h after being plated (1). To minimize the effects of endogenous cyclin D1 or other cell-cycle factors in our studies, overexpression experiments were performed during the first 30 h after hepatocyte isolation.
At this time, cells are in mid-G1 phase (27) and produce relatively low levels of cyclin D1 (Fig. 3A).

Surprisingly, cyclin D1 overexpression only slightly increased basal apoptosis; however, it significantly augmented the ability of DCA to induce apoptosis ($P < 0.01$; Fig. 3B). Decreasing cyclin D1 levels by culturing cells in the absence of serum did not significantly reduce DCA-induced cell death (data not shown), suggesting that other factors may play a role. UDCA and TUDCA alone induced a moderate degree of apoptosis in cyclin D1-overexpressing cells ($P < 0.01$). In addition, UDCA and TUDCA were ~50% less efficient at reducing DCA-induced apoptosis when cyclin D1 was overexpressed. Similarly, the ability of UDCA and TUDCA to decrease cyclin D1 expression alone or in combination with DCA was significantly impaired when the protein was overexpressed by the adenovirus (Fig. 3C). These results suggest that down-regulation of cyclin D1 is important in UDCA and TUDCA promotion of cell survival.

**Bax translocation and cytochrome c release are amplified by cyclin D1.** It was important to better define the mechanisms of apoptosis in hepatocytes with bile acids and/or cyclin D1 overexpression. Although not statistically significant, total Bax protein levels were slightly increased as a result of DCA incubation in both ADV-βgal- and ADV-D1-infected cells (Fig. 4A). Nevertheless, our results indicated that DCA induced Bax translocation ($P < 0.05$), which in turn was prevented by UDCA and TUDCA ($P < 0.05$; Fig. 4B). Surprisingly, cyclin D1 overexpression resulted in Bax translocation when compared with ADV-βgal-expressing cells ($P < 0.05$). DCA treatment additionally increased Bax translocation in cyclin D1-infected hepatocytes. Importantly, UDCA and TUDCA were less efficient at protecting against DCA’s effects. Data on cytochrome c release into the cytosol were in agreement with Bax changes (Fig. 4B).

**Cyclin D1-induced Bax translocation is p53 dependent.** It is thought that cyclin D1-induced apoptosis occurs through either p53-dependent or -independent mechanisms (13). p53, in turn, may induce apoptosis by modulating multiple pro- and antiapoptotic gene products (41) and Bax translocation to the mitochondrial membrane (11). In addition, we have previously shown (36) that UDCA specifically inhibits the E2F-1/p53/Bax apoptotic pathway in primary rat hepatocytes. Neither p53 total...
levels nor nuclear trafficking were significantly affected by bile acids compared with controls. Furthermore and as predicted, p53 expression levels under basal conditions or following bile acid incubation were very low (data not shown). Nevertheless, to further investigate the role of p53, primary rat hepatocytes were transfected with p53 siRNA and infected with either ADV-βgal or ADV-D1 in the presence or absence of bile acids. By Western blot analysis, p53 siRNA suppressed expression of p53 by 65% compared with control cells transfected with an irrelevant siRNA (Fig. 5A). Furthermore, cyclin D1 protein levels were slightly reduced in ADV-D1-infected hepatocytes with silencing. Nevertheless, modulation of cyclin D1 by bile acids was not significantly affected by p53 siRNA transfections in both ADV-βgal- and ADV-D1-infected cells (data not shown). Apoptosis was markedly reduced in p53 siRNA-transfected cells (Fig. 5B). In addition, cyclin D1 overexpression no longer decreased UDCA and TUDCA inhibition of DCA-induced apoptosis after p53 gene silencing. Similar results were obtained by using LDH viability assays (data not shown). Thus p53 plays a key role during cyclin D1-mediated apoptosis by bile acids. Interestingly, Bax translocation by bile acids was also inhibited and was not significantly different between ADV-βgal and ADV-D1-infected cells following transfection with p53 siRNA (Fig. 5C). Inhibition of Bax translocation in p53 siRNA-transfected cells did not completely inhibit apoptosis induced by DCA, suggesting that additional mechanisms contribute to apoptosis by bile acids.

**DISCUSSION**

The mechanisms of bile acid effects on cell survival and apoptosis are not entirely understood. We have previously reported that UDCA is able to prevent apoptosis in primary rat hepatocytes by inhibiting the mitochondrial pathway (28, 30) and modulating the E2F-1/p53/Bax apoptotic cascade (36). In addition, we have recently shown that UDCA and TUDCA specifically regulate cell cycle-related genes, including cyclin D1 (4). Hydrophobic bile acids such as DCA may either promote cancer growth or induce cell death and apoptosis (21). DCA increases cyclin D1 mRNA and protein expression in both primary rat hepatocytes and colon cancer cell lines (17, 23). Our results address the mechanisms by which bile acids modulate apoptosis upstream of mitochondrial commitment and demonstrate that cyclin D1 facilitates apoptosis of primary rat hepatocytes and is modulated differentially by bile acids. In fact, DCA exposure resulted in cyclin D1 expression and cell death, but preincubation with UDCA and TUDCA significantly abrogated both effects.

The role of cyclin D1 as a facilitator of hepatocyte apoptosis was further established by cyclin D1 overexpression. Previous studies have already suggested that increased expression of cyclin D1 initiates apoptotic events that are, however, incapable of culminating in cell death (26). In fact, cyclin D1 overexpression per se only marginally increased basal levels of apoptosis but significantly amplified DCA-induced apoptosis while decreasing UDCA and TUDCA cytoprotection. Our results suggested that UDCA and TUDCA downregulate cyclin...
D1 expression at the transcriptional level, possibly explaining their reduced antiapoptotic properties after cyclin D1 overexpression. Importantly, although significantly reduced, the antiapoptotic properties of UDCA and TUDCA were not completely abolished, indicating that they are only partially dependent on cyclin D1.

Although the role of cyclin D1 in proliferation is well established, less is known about its function in promoting apoptosis. It is thought that cyclin D1-induced apoptosis may occur through several distinct mechanisms (13). Elevated cyclin D1 levels could eliminate retinoblastoma protein function through its functional inactivation, releasing free E2F-1, which directly or in combination with other proteins may activate genes necessary for apoptosis (9). In that sense, we have previously shown that TGF-β1-induced apoptosis is associated with activation of E2F-1, which in turn is inhibited by UDCA (36). Other studies (22, 26) suggest that cyclin D1 may also amplify stress-induced mitochondrial pathways of apoptosis. Here we confirmed that UDCA and TUDCA prevented DCA-induced Bax translocation and cytochrome c release. Interestingly, cyclin D1 overexpression amplified DCA-induced Bax translocation and cytochrome c release and reduced UDCA and TUDCA cytoprotection. In addition, cyclin D1 overexpression alone increased Bax translocation and cytochrome c, without significantly altering apoptosis. Similar results have been reported in other models of cyclin D1 overexpression (26) and, although not completely understood, they suggest that in the absence of an additional toxic stimuli, endogenous levels of
immunoblots are shown. Densitometry analysis of at least 3 independent experiments. Representative
incubated with 50 μM DCA for 8 h. In coinoculation experiments, cells were pretreated with 100 μM UDCA or TUDCA 1 h before incubation with DCA. Cells were infected with ADV-D1 or ADV-βgal 16 h before addition of DCA. In addition, cells were transfected with either control or p53 siRNA 4 h before adenoviral infection. Cells were fixed and stained for morphological evidence of apoptosis. In addition, total proteins were extracted and subjected to Western blot analysis of p53 and cyclin D1. Total, mitochondrial, and cytosolic proteins were isolated for Bax analysis as described in MATERIALS AND METHODS. Fold changes were calculated based on densitometry analysis of at least 3 independent experiments. Representative immunoblots are shown. A: p53 and cyclin D1. B: percentage of apoptosis. C: total and mitochondrial Bax in cells incubated with bile acids and transfected with p53 siRNA in the presence or absence of ADV-βgal or ADV-D1 (top) and histogram of Bax mitochondrial levels in cells incubated with bile acids and transfected with control or p53 siRNA in the presence or absence of ADV-βgal or ADV-D1 (bottom). Blots were normalized to cytochrome c oxidase II (Cox II) or β-actin. Results are expressed as means ± SE of arbitrary units of at least 3 different experiments. *P < 0.05 and †P < 0.01 from control; *P < 0.01 from corresponding control siRNA-transfected cells.

Bcl-2 antiapoptotic proteins are sufficient to inhibit cell death, even at a postmitochondrial level (8, 32). Alternatively, mitochondrial function may not be severely impaired in such cases (20).

Previous findings have shown that UDCA and TUDCA modulate the E2F-1/p53/Bax apoptotic pathway (34, 36). Thus we investigated whether modulation of cyclin D1-associated apoptosis by bile acids involved the p53 protein. Here we show that p53 total and nuclear levels were very low and only slightly modulated by either bile acids or cyclin D1 overexpression, stressing the importance of UDCA’s and TUDCA’s effects on mitochondria, as previously described (28). Importantly, modulation of apoptosis by DCA through cyclin D1 appears to depend on p53. Gene silencing of p53 completely abrogated cyclin D1’s effects in Bax translocation, cytochrome c release, and apoptosis, despite the elevated levels of cyclin D1. Although the precise mechanism remains unclear, it conceivably involves increased cyclin D1 translocation to the nucleus or, alternatively, inhibition of its nuclear export (37). Although moderate levels of nuclear cyclin D1 are usually responsible for cell-cycle progression, higher levels may generate sufficient genetic instability that results in activation of p53 and apoptosis. With transactivation of cyclin D1, DCA may be amplifying the latter effect, whereas UDCA and TUDCA are effective at inhibiting it. Interestingly, we have shown that UDCA reduces E2F-1 transcriptional activation and p53 stabilization induced by TGF-β1 (36). Therefore, cyclin D1 may represent an alternative, upstream target of this pathway by activating E2F-1.

In addition, hepatocytes were still able to undergo DCA-induced apoptosis in the absence of p53, although to a lesser extent. In fact, p53 gene silencing significantly inhibited overall Bax translocation to the mitochondria. This implies the existence of both p53-dependent and -independent signals modulated by bile acids, and in fact Fas-mediated signaling, for example, is thought to be involved in bile-acid induced liver injury (14).

The mechanism by which p53 modulates cyclin D1-induced Bax translocation and apoptosis remains to be determined, because p53 protein levels were relatively unchanged. Nevertheless, p53 is highly governed through complex networks of posttranslational modifications (39) and may even promote cell death by direct interaction with the mitochondria (18, 31). This may result in the activation of Bax in the cytoplasm (7).

Finally, p53 is known to transcriptionally activate several p53-responsive genes, including Noxa and Puma (2), which may act in a Bid-like fashion to facilitate Bax activation. The induction of cyclin D1 can additionally be mediated by a target of p53, the p21 (WAF1/CIP1) inhibitor of cyclin-dependent kinases (6). In this regard, it was recently shown that p21 potentiates bile acid-induced p53-dependent apoptosis in hepatocytes (27). Therefore, further studies are necessary to determine whether modulation of Puma, Noxa, p21, or other proteins are responsible for cyclin D1 amplification of bile acid-induced apoptosis. These studies would also benefit our current understanding about bile acid function in cell proliferation. In fact, the (patho)physiological significance of our data may be more relevant considering different genetic backgrounds. DCA has been shown to use the β-catenin-cyclin D1 pathway to promote colon cancer (23). On the other hand, UDCA has been shown to have a chemopreventive role in a rat model of colon...
cancer by inhibiting azoxymethane-induced cyclin D1 (40). Therefore, cyclin D1 may provide the link between the apparently conflicting roles of bile acids in cell proliferation and apoptosis.

Collectively, our studies suggest that DCA-induced mitochondrial apoptosis is associated with and amplified by cyclin D1. UDCA and TUDCA inhibition of cyclin D1 expression contribute to their antiapoptotic effects. Furthermore, bile acid modulation of apoptosis through cyclin D1 is associated with p53-dependent Bax translocation to the mitochondria. The identification and characterization of upstream apoptotic targets for bile acids may result in the development of novel therapeutic options for diseases associated with deregulated apoptosis.

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