Synergistic Effects of 8-Chlorocyclic-AMP and Retinoic Acid on Induction of Apoptosis in Ewing’s Sarcoma CHP-100 Cells

Rakesh K. Srivastava, Aparna R. Srivastava, and Yoon S. Cho-Chung

Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology [R. K. S., Y. S. C.-C.], Medicine Branch [A. R. S.], National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

The enhanced expression of the regulatory subunit of cyclic AMP (cAMP)-dependent protein kinase type I, RIα, has been correlated with cancer cell growth. Retinoic acid (RA) has been shown to play an important role in the regulation of proliferation and differentiation in neoplastic cells. In the present study, the effects of cAMP analogue 8-chlorocyclic-AMP (8-Cl-cAMP) and RA (both singly and combined) on growth inhibition and apoptosis in Ewing’s sarcoma CHP-100 cells were evaluated. The inhibitory effects of 8-Cl-cAMP and RA (9-cis-RA, 13-cis-RA, and all-trans-RA) on cell viability were time and dose related. The degree of growth inhibition induced by 9-cis-RA was the greatest among all of the RA analogues (13-cis-RA and all-trans-RA) examined. The combined effects of 8-Cl-cAMP and RA on the induction of growth arrest at the G0/G1 stage of the cell cycle, apoptosis, down-regulation of RIα, and cleavage of poly(ADP-ribose) polymerase were synergistic. In conclusion, it is clear that RA and 8-Cl-cAMP act in a synergistic fashion and have potential for combination chemotherapy for the treatment of malignant disease.

INTRODUCTION

The actions of cAMP are well known in the regulation of various cellular functions including cell proliferation, differentiation, and gene induction through the activation of cAMP-dependent PKA (1). There are two types of PKA, PKA-I and PKA-II, which share a common catalytic subunit but contain different R subunits, RI and RII, respectively (2). Through biochemical studies and gene cloning, four isoforms of the R subunits, RIα, RIβ, RIIα, and RIIβ, have been identified (3, 4). Differential expression of PKA-I and PKA-II has been correlated with cell differentiation and neoplastic transformation (5, 6). RI/PKA-I is preferentially expressed in transformed cells or during the early stages of ontogenesis, whereas expression of RII/PKA-II is induced in cancer cells growth-arrested after treatment with cAMP analogues or differentiating agents (5–7). Thus, the dual signals, positive and negative, transduced by cAMP may depend on the availability of RI and RII subunits, respectively. A site-selective cAMP analogue, 8-Cl-cAMP, down-regulates RI/PKA-I and up-regulates RII/PKA-II in several cancer cell lines (6). It has been hypothesized (6) that RIα is an onogenic growth-inducing protein, and its constitutive expression disrupts normal ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy. Subsequently, it has been shown using an antisense strategy that RIα has a role in neoplastic cell growth in vitro (8, 9) and in vivo (10).

Cancer chemoprevention by agents that delay, reverse, or block cancer development has received much attention recently (11). Both natural and synthetic retinoids are prominent chemopreventive agents that have strong antiproliferative activity against certain types of cancers (12, 13). Similarly, many tissue culture cells undergo differentiation in response to retinoid treatment. In addition, retinoids stimulate programmed cell death in cultured HL-60 human promyelocytes and neuroblasticoma cell lines (14, 15). A strong relationship between vitamin A and cancer development has been established by numerous investigations over the last couple of decades. It is believed that physiological levels of retinoids guard the organism against the development of premalignant and malignant lesions.

Retinoids exert their modulatory effects on cell growth by binding to the retinoid receptor nuclear proteins, of which there are two classes, the RARs and the RXRs, each of which has three subtypes (α, β, and γ). The RARs form heterodimers with the RXRs, which enhance their gene transcriptional responses (16, 17). The retinoid receptors may influence gene transcriptional activation by binding to specific DNA sequences (retinoid response elements, i.e., RA response elements and retinoid X response elements; Ref. 18). Because RAR-β contains cAMP response element, PKA may modulate the responsiveness of RAR-β to RA.

Apoptosis is an active and gene-directed form of cell death with well-characterized morphological and biochemical features (19). The DNA repair enzyme PARP is known to be degraded during apoptosis (20, 21). However, cleavage of PARP has not been demonstrated during RA-induced apoptosis.

In advanced or recurrent malignant diseases, the development of adjuvant chemotherapy has improved response rates, but the overall impact on survival has been minimal. Moreover, chemotherapeutic treatment is complicated by toxicity as well as by the development of drug resistance. Thus, the development of new chemotherapeutic agents and new combination regimens is highly desirable. The purpose of this paper was to identify
conditions that yield highly favorable interactions between 8-CI-cAMP and RA (9-cis-RA, 13-cis-RA, or all-trans-RA) that induce apoptosis. The results from this study will serve as a basis (drug ratios and concentrations) for later in vivo analyses, which will be more predictive of clinical responses to these two agents in combinations.

MATERIALS AND METHODS

Reagent. RAs (9-cis-RA, 13-cis-RA, and all-trans-RA) were purchased from Sigma Chemical Co. (St. Louis, MO). RAs were dissolved in ethanol to a concentration of 1 mM, stored at −20°C, and protected from light. Anti-PARP antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An enhanced chemiluminescence Western blot detection kit was purchased from Amersham Life Sciences, Inc. (Arlington Heights, IL). The protein concentration was determined with the BCA reagent (Pierce, Rockford, IL).

Cell Viability Assay. Human Ewing’s sarcoma CHP-100 cells were obtained from Dr. Len Neekers (National Cancer Institute, Bethesda, MD). Cells (1 × 10⁴ cells/well) were plated in a 24-well plate containing RPMI 1640 supplemented with 10% FCS and 1% antibiotics at 37°C with 5% CO₂. RA (9-cis-RA, 13-cis-RA, and all-trans-RA) was added (8 h after seeding) to four replicate wells at varying concentrations (see Fig. 1 legend). Viable cells were quantitated by MTT assays. On the indicated days, 20 ml of MTT solution (5 mg/ml) were added for 3 h to each well, followed by the addition of 100 ml of 10% SDS and 0.01 mM HCl. Culture medium was transferred to a 96-well plate just before reading the absorbance. Viability was quantitated by measuring absorbance at 570 nm, using an ELISA plate reader with a reference wavelength of 650 nm. The mean absorbance of four cultures/time point is plotted.

Soft Agar Growth. Cells (10,000) were seeded in 1 ml of 0.3% DIFCO Noble agar (DIFCO) in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotics). This suspension was layered over 1 ml of 0.8% agar medium base layer in 12 multiwell cluster dishes (Becton Dickinson), and after 7 days, cells were stained with nitro blue tetrazolium (Sigma) overnight. Colonies larger than 0.2 mm were counted with an Artek 880 colony counter (Artek Systems).

Photoaffinity Labeling Followed by Immunoprecipitation of R Subunit. Cells were washed twice with ice-cold PBS before harvesting. The cell pellets were suspended in 0.5 ml of lysis buffer [0.1 mM NaCl, 5 mM MgCl₂, 1% NP40, 0.5% sodium deoxycholate, and 20 mM Tris-HCl (pH 7.4)] containing proteolysis inhibitors, vortexed, passed through a 22-gauge needle twice, allowed to stand for 30 min at 4°C, and centrifuged at 750 × g for 20 min; the resulting supernatants were used as cell lysates. Lysates were electrophoresed on SDS-PAGE, blotted onto nitrocellulose membrane, probed with anti-PARP antibody, and visualized by the enhanced chemiluminescence kit.

Cell Morphology and Apoptosis. To examine whole cell morphology, cells were washed with PBS, fixed with 70% methanol for 5 min, and stained with Giemsa (Bio-Rad) for 15 min. After staining, the whole cells were visualized under an inverted microscope. To assay nuclear morphology (apoptotic nuclei), cells were washed with PBS, fixed with 70% ethanol for 1 h, and stained with 1 μl Hoechst 33258 (Sigma) for 30 min. The nuclear morphology of the cells was visualized by a fluorescence microscope (Olympus BH2). Fluorescent nuclei were screened for normal morphology (unaltered chromatins), and apoptotic nuclei comprising those with fragmented (scattered) and condensed chromatins were counted. Apoptosis was expressed as the percentage of apoptotic nuclei/10⁵ nuclei.

Nucleosome ELISA Assay. The nuclease system ELISA allowed the quantitation of apoptotic cells in vitro by DNA affinity mediated by capture of free nucleosomes followed by their anti-histone-facilitated detection. In this assay, mono- and oligonucleosomes were captured on precoated DNA-binding proteins. Anti-histone 3 biotin-labeled antibody then bound to the histone component of captured nucleosomes and was detected after incubation with streptavidin-linked horseradish peroxidase conjugate. Horseradish peroxidase catalyzed the conversion of colorless tetramethylbenzidine to blue. The addition of stop solution changed the color to yellow, the intensity of which was proportional to the number of nucleosomes in the sample. CHP-100 cells (1 × 10⁶) were seeded into a 24-well plate. After cells attached to the plate (0 h), RA (9-cis-RA, 13-cis-RA, and all-trans-RA) were added, or without 8-Cl-cAMP was added. After 5 days, cells were harvested for nuclease ELISA assay as per the manufacturer’s directions (Oncogene Research Products, Cambridge, MA).

Cell Cycle Analysis. CHP-100 cells were seeded into a 10-cm-diameter plate and cultured to confluence. Confluent cells were harvested and reseeded at 1 × 10⁶ cells/plate. Cells were treated with drugs, cultured for 5 days, and harvested for cell cycle analyses. In brief, 1 × 10⁶ cells were washed in ice-cold PBS and pelleted in a 12 × 75-mm culture tube. The pellets were resuspended in 0.875 ml of ice-cold PBS, and 0.125 ml of ice-cold 2% paraformaldehyde solution was added. The tubes containing the cells were incubated at 4°C for 1 h and centrifuged for 5 min at 250 × g, and the supernatant was discarded. After fixation, cells were permeabilized by adding 1 ml of 0.2% Tween 20 in PBS at room temperature and incubated for 15 min at 37°C. Cells were washed by adding an additional 1 ml of PBS and centrifuged. After aspiration of the supernatant, cells were resuspended in 1 ml of PBS containing 4',6-diamidino-2-phenylindole at a final concentration of 1 μg/ml for 30 min. The samples were read on a FACStar flow cytometer.
RESULTS

Synergistic Inhibitory Effects of 8-Cl-cAMP and RA on Cell Viability and Colony Growth. To evaluate the responsiveness of CHP-100 to either RA or 8-Cl-cAMP, we first analyzed the viability of cells as determined by the MTT assay to increasing concentrations of either 8-Cl-cAMP or RA. Treatment of the cells with cAMP analogue 8-Cl-cAMP inhibited viability in dose- and time-dependent manners (Fig. 1A). Similarly, 9-cis-RA, 13-cis-RA, or all-trans-RA resulted in the reduction of cell viability in a dose-dependent manner (Fig. 1B). The 9-cis-RA was more potent than either 13-cis-RA or all-trans-RA in inhibiting cell viability. Because both 8-Cl-cAMP and RA were effective in inhibiting viability, we next examined the interactive effects of 8-Cl-cAMP and RA on growth inhibition. A low dose of 8-Cl-cAMP acted synergistically with 9-cis-

(Becton Dickinson Immunocytometry Systems, Mountain View, CA) using the Lysys II software program.

**Fig. 1** Viability of CHP-100 cells to 8-Cl-cAMP and RA (9-cis-RA, 13-cis-RA, or all-trans-RA) alone and in combination. A, dose-dependent inhibition of cell viability by 8-Cl-cAMP. Cells were treated with 8-Cl-cAMP for 1, 3, and 5 days. Data (mean ± SE of quadruplicate determinations) represent one of three separate experiments that gave similar results. B, dose-dependent inhibition of cell viability by RA (1 nM to 100 μM). Cells were treated with 9-cis-RA, 13-cis-RA, or all-trans-RA for 5 days. Data (mean ± SE of quadruplicate determinations) represent one of three separate experiments that gave similar results. C, interactive effects of RA (9-cis-RA, 13-cis-RA, and all-trans-RA) and 8-Cl-cAMP on cell viability. Cells were treated with 9-cis-RA (1 nM), 13-cis-RA (1 nM), or all-trans-RA (1 nM) with or without 8-Cl-cAMP (1 μM) for 5 days. Data (mean ± SE of quadruplicate determinations) represent one of three separate experiments that gave similar results.

**Fig. 2** Synergistic effect of 8-Cl-cAMP and 9-cis-RA on growth inhibition. Cells were treated with 8-Cl-cAMP (1 μM) and 9-cis-RA (1 nM) singly and in the presence of various concentrations of the other drug at day 0, and the cell number was counted at day 5. The data are expressed as the percentage of growth inhibition in reference to the growth of untreated control cells. The striped portion of the bars represents the percentage growth inhibition values for 1 μM 8-Cl-cAMP (A) and 1 nM 9-cis-RA (B) when added alone. The open portion of the bars represents the percentage growth inhibition values for increasing concentrations of 9-cis-RA (A) and 8-Cl-cAMP (B) when added alone. The height of the bars on the left of each pair represents the sum of the individual drug effects or the expected percentage growth inhibition if analogues were added together. The total heights of the solid bars indicates the observed percentage of growth inhibition when drugs were added in combination at the indicated concentrations. The differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition. Data (mean ± SE of quadruplicate determinations) represent one of three separate experiments that gave similar results. The insets show the synergism quotient at each concentration of analogue combination. The synergism quotient was defined as the net growth-inhibitory effect of a drug combination divided by the sum of the net individual drug effects on growth inhibition. 8-Cl, 8-Cl-cAMP, 9-cis, 9-cis-RA.
RA, 13-cis-RA, or all-trans-RA in inhibiting the viability of CHP-100 cells (Fig. 1C). We next examined the synergism quotients of interactive effects of 8-Cl-cAMP and 9-cis-RA by keeping the concentration of one drug constant and varying the concentration of other drug and vice versa (Fig. 2, A and B). The combination of 1 \mu M 8-Cl-cAMP and 1 nm RA yielded the largest synergistic effect (Fig. 2, A and B).

Anchorage-independent growth has been widely used as one of the criteria to establish the transformed phenotype in cells. We examined the interactive effects of 8-Cl-cAMP and RA together on colony formation by growing the cells in soft agar (Fig. 3). The 8-Cl-cAMP acted synergistically with 9-cis-RA, 13-cis-RA, or all-trans-RA in inhibiting colony (0.2 and 1 mm in diameter) formation (Fig. 3).

**Synergistic Effects of 8-Cl-cAMP and RA on Down-Regulation of RI\alpha Protein.** We next determined the effects of 8-Cl-cAMP and RA on the levels of R subunits in the CHP-100 cell extracts by photoaffinity labeling with 8-N3-[32P]cAMP and immunoprecipitation. The photoaffinity labeling showed that 8-Cl-cAMP or RA (9-cis-RA, 13-cis-RA, or all-trans-RA) significantly decreased the level of RI\alpha protein on day 5 (Fig. 4). The RI\alpha protein in the cell extracts was further identified by immunoprecipitation with anti-RI\alpha antiserum (kindly provided by S. D. Park, Seoul National University, Seoul, Korea), and the immunoprecipitated proteins were resolved by SDS-PAGE (Fig. 4). Low doses of RA (1 nm) or 8-Cl-cAMP (1 \mu M) had little or no effects on RI\alpha levels (Fig. 4). Interestingly, combined treatment of 8-Cl-cAMP (1 \mu M) with 9-cis-RA, 13-cis-RA, or all-trans-RA RA (1 nm) resulted in the down-regulation of RI\alpha protein (Fig. 4). 8-Cl-cAMP and RA either singly or in combination did not alter the RI\alpha protein level (Fig. 4).

**Synergistic Effects of 8-Cl-cAMP and RA on Cell Morphology and Apoptosis.** The morphology of CHP-100 cells exposed to 8-Cl-cAMP and RA (9-cis-RA, 13-cis-RA, or all-trans-RA) was examined by staining with Giemsa (Fig. 5A). When cells were evaluated for morphology, only combined treatment of RA and 8-Cl-cAMP displayed a unique morphology that is different from untreated control cells. Single treatment of either RA or 8-Cl-cAMP did not produce a change in morphology (Fig. 5A). By comparison, both RA (9-cis-RA, 13-cis-RA, or all-trans-RA)- and 8-Cl-cAMP-treated cells exhibited changes in cell morphology (elongated shape, increased cytoplasm:nucleus ratio, and flat phenotype) or apoptosis (round-shaped cells, chromatin condensation, and nuclear fragmentation) and grew sparingly to form much smaller cell clusters (Fig. 5, A and B). Although ineffective alone, 8-Cl-cAMP and RA (9-cis-RA, 13-cis-RA, or all-trans-RA) together had synergistic effects on apoptosis (Fig. 5C).

**Synergistic Effects of 8-Cl-cAMP and RA on the Induction of PARP Cleavage and Nucleosome Release.** In recent years, PARP activation has been used as an index of apoptosis induced by variety of anticancer drugs. Therefore, we measured the activation of PARP to confirm that cells were undergoing apoptosis due to drug treatment. The effects of 8-Cl-cAMP and RA, alone and in combination, on PARP cleavage were examined in CHP-100 cells (Fig. 6A). Combined treatment of 8-Cl-cAMP (1 \mu M) and RA (9-cis-RA or all-trans-RA; 1 nm) resulted in cleavage of p116PARP to its 85-kDa fragment (Fig. 6A). By comparison, cleavage of PARP was not observed in cells treated with 8-Cl-cAMP and RA, alone.
with low doses of 8-Cl-cAMP or RA or in untreated control cells (Fig. 6A).

We further confirmed the induction of apoptosis caused by RA and/or 8-Cl-cAMP by conducting a nucleosome ELISA assay (Fig. 6B). As expected, treatment with RA and 8-Cl-cAMP together caused an increase in free nucleosomes as compared to treatment with RA or 8-Cl-cAMP alone (Fig. 6B). These results confirmed our above findings that RA and 8-Cl-cAMP act in a synergistic fashion in inducing apoptosis in breast cancer cells.

**Synergistic Effects of 8-Cl-cAMP and RA on the Cell Cycle Arrest at G0-G1.** Because 8-Cl-cAMP and RA inhibited cell viability, it was of interest to examine whether they caused cell cycle arrest. RA and 8-Cl-cAMP alone had little effect on growth arrest at the G0-G1 stage of the cell cycle (Table 1). Interestingly, combined treatment of 8-Cl-cAMP (1 μM) and RA (9-cis-RA, 13-cis-RA, or all-trans-RA; 1 nM) caused synergistic effects on growth arrest at the G0-G1 stage of the cell cycle.

**DISCUSSION**

In the present study, we have demonstrated that the apoptosis and phenotype of Ewing’s sarcoma CHP-100 cells can be regulated by the combined treatment with cAMP analogue 8-Cl-cAMP and RA. Furthermore, these processes were concentration dependent and synergistically activated by the combination of 8-Cl-cAMP and RA. The degree of growth inhibition induced by 9-cis-RA was the greatest among all RA (13-cis-RA and all-trans-RA) analogues examined. The combination of 8-Cl-cAMP and RA resulted in cell growth inhibition that was correlated with down-regulation of the RIα protein, induction of phenotypic change, apoptosis (PARP cleavage and nucleosome release), and cell cycle arrest at G0-G1. This is the first report showing that the combined treatment of 8-Cl-cAMP with RA suppressed growth by inducing apoptosis.

The most important impact of this investigation is the observation that RA enhances the growth-inhibitory effects of 8-Cl-cAMP on CHP-100 cells in a dose-dependent manner. Comparison of growth inhibition by RA or 8-Cl-cAMP alone versus a combination of these two agents showed a substantial enhancement of growth inhibition at clinically relevant concentrations. Based on present study, it is clear that these drugs act in a supra-additive or synergistic fashion and have potential for combination chemotherapy.

Many of the effects of retinoids result from the modulation of gene expression. Nuclear retinoid receptors, ligand-activated transcription-enhancing factors, play a major role in mediating the effects of retinoids on gene expression and consequently on the growth and differentiation of both normal and tumor cells (22). Two types of nuclear retinoid receptors, RARs and RXRs, have been identified. These receptors exhibit distinct ligand binding properties; the RARs bind all-trans-RA and 9-cis-RA, whereas the RXRs bind 9-cis-RA selectively. The RXRs and RARs form heterodimers that bind to specific DNA sequences, called RA response elements, and enhance the transcription of retinoid-responsive genes. Changes in the expression of specific receptors could abrogate the retinoid signaling pathway and result in enhanced carcinogenesis. In our study, 8-Cl-cAMP may synergize the effects of RA by enhancing the expression of RAR-β2, because RAR-β2 promoter has a cAMP-response element (23).

The role of protein kinases has been studied in cell cycle
Additional information and analysis related to cell cycle regulation and apoptosis by synergistic effects of 8-Cl-cAMP and RA.

**Fig. 6** Effects of 8-Cl-cAMP and RA treatment on cleavage of PARP and release of nucleosomes. **A**, PARP levels were measured in cell lysates by Western blot analysis. **B**, free nucleosome release measured by the nucleosome ELISA assay. CHP-100 cells were treated with 8-Cl-cAMP (1 μM) and RA (9-cis-RA, all-trans-RA, or 13-cis-RA; 1 nM), alone or in combination, for 5 days. Data (mean ± SE of quadruplicate determinations) represent one of three separate experiments that gave similar results.

**Table 1** Effects of 8-Cl-cAMP and RA (9-cis-RA, 13-cis-RA, and all-trans-RA) treatment on the cell cycle in CHP-100 cells. Cells were treated with 8-Cl-cAMP and RA alone and in combination for 5 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G₀-G₁ phase</th>
<th>S phase</th>
<th>G₂-M phase</th>
</tr>
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<tr>
<td>Control</td>
<td>40.2</td>
<td>28.9</td>
<td>30.9</td>
</tr>
<tr>
<td>9-cis-RA (1 nM)</td>
<td>57.3</td>
<td>30.8</td>
<td>11.9</td>
</tr>
<tr>
<td>13-cis-RA (1 nM)</td>
<td>51.3</td>
<td>37.4</td>
<td>11.4</td>
</tr>
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<td>All-trans-RA (1 nM)</td>
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<td>40.1</td>
<td>15.9</td>
</tr>
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and leading to apoptosis/differentiation. In HL-60 cells, RIₜ antisense triggered granulocytic differentiation (25), whereas in MDA-MB-231 breast cancer cells, RIₜ antisense triggered apoptosis (26).

In the present study, the combined effects of RA and 8-Cl-cAMP caused a decrease in the RIₜ protein level. Such a coordinated reduction in RIₜ due to combined treatment with RA and 8-Cl-cAMP has also been shown by us in breast cancer cells.³ Because RIₜ expression is enhanced in human cancer cell lines and primary tumors, it is a target for cancer diagnosis and therapy. Both apoptosis and differentiation were synergistically stimulated by the combination of RA and 8-Cl-cAMP. Targeting RIₜ through RA and 8-Cl-cAMP was associated with the induction of apoptosis.

Apoptosis is defined by characteristic morphological and biochemical changes (27). A multitude of factors modulate apoptosis induction, including growth factors, intracellular mediators of signal transduction, and nuclear proteins regulating gene expression, DNA replication, and the cell cycle (27). Apoptosis directly regulates tumorigenesis and could potentially be disrupted in tumor cells, conferring a survival advantage (27). In addition to proliferation, apoptosis is the major factor that contributes to the actual growth of a cell clone (28). Our findings demonstrate that combined treatment with 8-Cl-cAMP and RAs resulted in a synergistic induction of apoptosis as evident from nuclear morphology and PARP cleavage. Thus, combined treatment with RA and 8-Cl-cAMP turns on signals for the blockade of cancer cell survival, suggesting its therapeutic potential for malignancy in humans.

**REFERENCES**


Synergistic effects of 8-chlorocyclic-AMP and retinoic acid on induction of apoptosis in Ewing’s sarcoma CHP-100 cells.

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