Identification of a Mutant Human Topoisomerase I with Intact Catalytic Activity and Resistance to 9-Nitro-camptothecin*

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Human U-937 myeloid leukemia cells were selected for resistance to increasing concentrations of the camptothecin derivative, 9-nitro-20(S)camptothecin (9-NC). The isolated single cell clone, designated U-937/CR, was approximately 20-fold resistant to 9-NC. Analysis of topoisomerase I (topo I) gene expression in U-937/CR cells demonstrated similar mRNA levels as compared with U-937 cells. Immunoblotting with an anti-topo I serum revealed reactive proteins at 100, 75, and 67 kDa which were expressed at the same level in the parental and 9-NC-resistant clones. These cell lines also demonstrated similar levels of topo I catalytic activity as determined by assaying nuclear extracts for relaxation of supercoiled plasmid DNA. In contrast, catalytic assays performed in the presence of 9-NC demonstrated that topo I activity from U-937/CR cells was approximately 10-fold more resistant than that from U-937 cells. Nucleotide sequencing of topo I cDNAs revealed the substitution of phenylalanine (TTC) at residue 361 in U-937 cells with serine (TCC) in the 9-NC-resistant clone. Expression and partial purification of the mutant topo I protein in Escherichia coli demonstrated resistance of this enzyme to 9-NC in catalytic assays. Taken together, these findings identify a novel mutation in topo I which confers resistance to 9-NC and support the involvement of this region in the interaction between topo I and 9-NC.

Topoisomerase I (topo I)† is a 100-kDa nuclear protein that relaxes transcription-associated duplex DNA (1–5). Topo I-mediated relaxation is accomplished by the introduction of a single-stranded nick in the phosphodiester backbone with passage of the intact DNA strand and relief of torsional strain. The creation of a single-stranded nick involves the formation of a covalent bond between the 3'-end of the DNA strand break and a tyrosine residue in topo I (4–6). The active site tyrosine of eukaryotic topo I has been mapped to a conserved domain near the carboxyl terminus of the enzyme (5, 6). Topo I also rejoins the nicked strand of DNA following relaxation. While religation would appear to involve the active site, it is not clear whether other domains of topo I are required for this event (7). Additional functions of topo I may involve a direct role in gene transcription. Studies have shown that topo I is required for transcription of supercoiled RNA gene in vitro (8) and that microinjection of anti-topo I antibodies into cells is associated with inhibition of transcription (9).

Topo I has been identified as a cellular target for the plant alkaloid camptothecin (CPT) (3, 10–12). The available evidence indicates that CPT binds to the topo I-DNA complex subsequent to the DNA cleavage step. While labeled CPT interacts reversibly with this complex, there is no detectable binding of this agent to isolated topo I or purified DNA (13, 14). Other studies have demonstrated that while CPT has little effect on topo I-mediated DNA cleavage, this agent inhibits the religation step (15, 16). Moreover, CPT has been found to preferentially stabilize topo I-mediated cleavage of T-G linkages following formation of the covalent bond between tyrosine and the 3'-phosphate group of thymidine (17). These findings have supported the availability of a CPT-binding site upon formation of the topo I-DNA complex. Although the nature of the CPT-binding site remains unknown, interaction of CPT and the topo I-DNA complex appears to be necessary for the cytotoxic effects of this agent. For example, while CPT inhibits topo I activity (10), the concentration necessary for such inhibition is considerably higher than that required for induction of cell lethality. Indeed, the available evidence supports a model in which CPT-induced stabilization of the topo I-DNA complex is associated with conversion of the single strand nicks to irreversible double strand breaks (18, 19).

Other studies have demonstrated that yeast cells devoid of topo I are resistant to the lethal effects of CPT (20, 21). Conversely, cells that overexpress topo I have been found to be hypersensitive to this agent (21, 22). These findings are in concert with the demonstration that mammalian cell lines selected for resistance to CPT exhibit decreased levels of topo I expression. In resistant murine P388 leukemia cells, CPT treatment is associated with decreased DNA single strand breaks compared to wild-type cells and decreased expression of topo I at the mRNA and protein levels (23). Similar findings have been obtained in hamster (24) and human (25, 26) cell lines. Decreases in topo I expression have been associated with rearrangement and hypermethylation of the topo I gene (27). Other studies demonstrating that activity of purified topo I from CPT-resistant cells is unimpaired in the presence of this agent have supported alterations in the enzyme that confer a resistant phenotype (25, 28, 29). In this context, several studies have identified mutations in the topo I gene in association with the development of CPT resistance. A point mutation resulting in replacement of threonine with alanine at residue 729 has been found in a CPT-resistant human lung cancer line (30), while changes at residues 533 and 583 have been identified in resistant human leukemia cells (31). Thus, decreased expres-
sion or alteration of the topo I gene can confer resistance to CPT.

Recent work has resulted in the synthesis of certain CPT
derivatives that are cytotoxic to tumor cells. For example, 9-ni-
tro-20'Scamptothecin (9-NC) inhibits the growth of human
carcinoma and melanoma cells in vitro and induces regression
of these tumors in immunodeficient mice (32–35). Other stud-
ies have demonstrated that exposure of human U-937 myeloid
leukemia cells to this agent is associated with induction of early
response gene expression and internucleosomal DNA fragmen-
tation (36). The present work describes the isolation of a U-937
cell clone selected for resistance to 9-NC. The results demon-
strate that these resistant cells exhibit similar levels of topo I
catalytic activity compared to that in parental cells and that
the topo I gene is mutated at a potential CPT-binding site in
the enzyme.

MATERIALS AND METHODS

Drugs—CPT and 9-NC were prepared and purified as described (37).
Both drugs were suspended in polyethylene glycol (PEG 400; Aldrich),
divided into small aliquots, and stored at –70 °C. Toposide was
obtained from Bristol-Myers (Evansville, IN).

Cell Culture—U-937 monoblastic leukemia cells (American Type
Culture Collection, Rockville, MD) were grown in RPMI 1640 media
containing 10% heat-inactivated fetal bovine serum, 100 unit/ml penicil-
lin, 100 mg/ml streptomycin, and 2 ml/l-glutamine. Resistant U-937
cells were maintained in the same media containing varying concen-
trations of 9-NC.

Cytotoxicity Assays—Drug cytotoxicity assays were performed as de-
scribed using the tetrazolium-based compound 3-(4,5-dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide (MTT) (38). Briefly, 100 µl of di-
luted drug or drug diluent alone was added to logarithmically growing
cells (2 × 105) in a 96-well plate. After incubation for 48 h, 50 µl of 3
mg/ml MTT in PBS were added and the cells incubated for an addi-
tional 3 h. Eighty µl of 25% SDS in 50% dimethylformamide
were then added and the plates incubated overnight at 37 °C to allow
solubilization of the formazan crystals. The absorbance values for each
well were determined in an enzyme-linked immunosorbent assay
reader (model MA300 Automated EIA Plate reader, Whittaker M. A.
Bioproducts, Inc., Walkersville, MD) at a wavelength of 550 nm. IC50
values were obtained by a linear regression analysis of percent absor-
bance versus log drug concentration.

RNA Isolation and Northern Blot Hybridization—Total cellular RNA
was isolated by a modification of the guanidine isothiocyanate tech-
nique (36). RNA (20 µg/lane) was separated by electrophoresis on
1% agarose, 2.5 unit/ml Moloney murine leukemia virus reverse tran-
scriptase, and 2.5 µl of random hexamers (Gene Amp RNA PCR Kit, Perkin Elmer
Cetus, Norwalk, CT). The mixture was successively incubated at 42 °C
for 15 min, at 99 °C for 5 min, and at 5 °C for 5 min. Oligonucleotide
primers of 25–35 base pairs were added at a final concentration of 0.15

**Table I**

| Pair Position | Sequences* |
|---------------|------------|
| 1 220–247, 968–993 | GGACCACCTCCAAACACAGTTCAAGATC, GGCTAGCCAAGAAAGAATGCACTTCCTCCCTTCG |
| 2 836–862, 1421–1447 | GGGCAGGGAGGCGCTATCTGGATAGGCTGAGTGG, GGCTGCCAGAAGAATGCACTTCCTCG |
| 3 1316–1341, 2274–2300 | GGAAATCTGACGTGCTGAGAGACGTAGCAAGTCAGC, GGCTGAGCAAGAAAGAATGCACTTCCTCG |
| 4 1991–2012, 2572–2596 | GGCCCGGGGCTTCAGACCCGGCGAAGCAGCAAGGGAGG |
| 5 2278–2307, 2782–2821 | GGCCCGGCGTCTCCAGACTGAGGAGAAAGCTGTGAGT, GGCTGAGCAAGAAAGAATGCACTTCCTCG |

* Numbered according to D'Arpa et al. (48).
* Restriction enzyme sites were added to the 5′-ends of some of the primers to facilitate future cloning.
μm and were designed to amplify regions of 500-1000 base pairs of a human topo I cDNA according to the GenBank model (Table I). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 394 using β-εanoethoxy phosphoramidite chemistry and were purified by ethanol precipitation before use. After addition of 2.5 units of Taq DNA polymerase, the amplification was performed at 95/72 °C using 30-s intervals for a total of 35 cycles. The products were analyzed by electrophoresis in a 1.5% agarose gel in TAE (0.04 mM Tris acetate, 1 mM ethylenediaminetetraacetate), and the amplified fragments were removed and purified from the gel using an affinity matrix (Glassmilk; Bio 101, Inc., La Jolla, CA). Approximately 1 ng of the purified fragment was sequenced by the dideoxynucleotide method using thermocycling and modified T7 DNA polymerase. Briefly, 10 pmol of one of the flanking primers or an internal primer were end-labeled with [γ-32P]ATP (New England Nuclear) and T4 polynucleotide kinase (Promega, Madison, WI). The primers were used in 6 μl of reaction mixtures containing Sequencing Grade Taq DNA polymerase and dNTP/ddATP mixtures (fmol Nucleic Sequence System, Promega). Thermal cycling was performed at 95/70 °C using 30-s intervals for 30 cycles. The products were analyzed in a 6% polyacrylamide gel containing 33 μM urea and TBE (0.1 M Tris-borate, 2 mM EDTA, pH 8.3). When electrophoresis was terminated, the gel was dried on filter paper and exposed for 16-48 h to Kodak film using an intensifying screen.

Expression of Mutant U-937 Topo I in E. coli—A human topoisomerase I expression plasmid was constructed by cloning the BamHI-EcoRI fragment of the plasmid ptac-topI1 (kindly provided by Dr. J. Wang (38)) into the vector pGEX-5X1 (Pharmacia LKB Biotechnology, Inc.). The resulting plasmid, pGEX-TOP1, contains a tac promoter controlling the expression of a fusion protein consisting of glutathione S-transferase (GST) linked to the amino terminus of human topo I. A plasmid expressing the mutant topo I was constructed by replacing the Ndel-BglII fragment in pGEX-TOP1 with a fragment corresponding to this region in the resistant topo I cDNA. The resultant plasmid, pGEX-MTOPI1, was sequenced to confirm the presence of the mutation. DH5α/F’IQ E. coli (Life Technologies, Inc.) infected with either pGEX-TOP1 or pGEX-MTOPI1 were grown in 50 ml of 2 X YT media containing 2% (w/v) glucose and 75 μg/ml ampicillin to an OD600 nm of approx. 0.5. After addition of isopropyl-β-D-thiogalactoside (IPTG) to 0.5 mM, the bacteria were grown for 2 h and harvested by centrifugation.

Lysetes were prepared by resuspension of the pellets in 2.5 ml of ice-cold PBS containing 0.1 mM PMSF and 10 μg/ml leupeptin, followed by sonication on ice. Triton X-100 was added to a concentration of 1% and the mixtures incubated on ice for 30 min. After centrifugation at 12,000 x g at 4 °C for 10 min, the supernatant was removed and incubated with 50 µl of a 50% (v/v) elution of Glutathione Sepharose 4B (Pharmacia LKB Biotechnology, Inc.) for 2 h at 4 °C. The beads were washed three times with 250 µl of PBS containing 0.1% PMSF and 10 µg/ml leupeptin. Bound proteins were eluted by incubation of the beads in 55 µl of elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0) for 10 min at room temperature. The elution step was repeated and the supernatants pooled. Glyceral was then added to a concentration of 30%, and the lysates were used immediately or stored at -20 °C. Topo I catalytic activity was assayed as described above, with the exception that reactions were performed for 60 min in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 mM KCl, and 15 μg/ml bovine serum albumin.

RESULTS

Development of 9-NC-resistant Cells—U-937 cells were selected for resistance to 9-NC by continuous exposure to an initial concentration of 20 nM drug for 2 weeks, and then growth for a similar period in media containing 40 nM 9-NC. Cells were subsequently exposed to 60, 80, 100, 125, and 150 nM 9-NC. Using this approach, a cell line, designated U-937-9CR (CR, camptothecin resistant) was isolated as a single cell clone capable of sustained growth in media containing 150 nM 9-NC. This resistant phenotype was stable after growth for 2 months in media lacking 9-NC. The growth rate of U-937/CR cells was similar to that of the parental U-937 cells with a doubling time of approximately 24 h. Furthermore, U-937/CR cells remained tumorigenic when tested as xenografts in nude mice (data not shown, (46, 47)).

Sensitivity of U-937 and U-937/CR cells to 9-NC was compared in MTT cytotoxicity assays. Parental U-937 cells were killed at an IC50 of approximately 30 nM 9-NC (Fig. 1). In contrast, 9-NC had little effect on U-937/CR cells at concentrations of 200 nM, and the IC50 for this line was nearly 700 nM. Similar results were obtained in three separate experiments (Table II). The finding that verapamil had no significant effect on the IC50 for 9-NC in U-937/CR cells supported the lack of P-glycoprotein involvement in this resistant phenotype (Table II). U-937/CR cells were also resistant to CPT, although the fold increase in IC50 compared with U-937 cells was less than that obtained with 9-NC (Table II). Taken together, these findings indicated that U-937/CR cells are resistant to 9-NC, as well as CPT; and that the mechanism of resistance may involve alterations in topo I expression.

Topo I Gene Expression—The basis for resistance of U-937/CR cells to 9-NC was studied by examining topo I gene expression at the mRNA and protein levels. Northern blot analysis of total cellular RNA from U-937 cells revealed 4.0-kb transcripts that hybridized to the topo I probe (Fig. 2). Similar findings were obtained with RNA from U-937/CR cells (Fig. 2). Moreover, intensity of the topo I signals was comparable for both lines (Fig. 2). The finding that the actin hybridization signals were similar for both lines confirmed equal loading of the lanes (Fig. 2).

Using polyclonal anti-topo I serum, three major immunoreactive proteins were detectable at approximately 100, 75, and 68 kDa in U-937 nuclear extracts (Fig. 3A). A similar pattern was observed with extracts of U-937/CR cells, and the intensity of the signals was comparable for both lines (Fig. 3A). Analysis of duplicate preparations by Coomassie Blue staining indicated equal loading of protein (Fig. 3A). Since previous studies have demonstrated that CPT resistance is associated with increases in topo II expression (48), we also reprobed the filters with a topo II antis serum. Topo II protein levels were increased 1.9-fold compared with the parental line (Fig. 3B). Taken together with the Northern analyses, these results demonstrate that topo I expression in the resistant U-937/CR phenotype is indistinguishable from that in U-937 cells. Moreover, the 9-NC-resistant phenotype is associated with increased topo II expression.

Topo I Catalytic Activity—Catalytic activity of topo I in U-937 and U-937/CR cells was determined by a plasmid relaxation assay performed in the absence of ATP (43). In this assay, varying amounts of nuclear protein were incubated with supercoiled plasmid DNA. In the absence of 9-NC, plasmid relaxation activity of nuclear extracts from U-937 cells was similar to that obtained with extracts from U-937/CR cells (Fig. 4A). Quantitation of the supercoiled bands by densitometry confirmed similar levels of activity in U-937 and U-937/CR cells (Fig. 4B). These results indicated that the resistant U-937/CR
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**FIG. 2. Analysis of topo I mRNA levels in U-937 and U-937/CR cells.** Total cellular RNA (20 μg) was isolated by the guanidine isothiocyanate technique, separated by electrophoresis in a 1% agarose gel stained with Coomassie Blue, and then transferred to nitrocellulose filters. The filters were hybridized to the 32P-labeled topo I cDNA from both cell lines. A. The filters were hybridized to the 32P-labeled topo I cDNA from both cell lines. B. The filters were hybridized to the 32P-labeled actin cDNA from both cell lines.

Other assays of topo I catalytic activity were performed in the presence of 9-NC. Under these conditions, the addition of 50 μM 9-NC resulted in nearly complete inhibition of the plasmid relaxation activity in U-937 cell extracts (Fig. 5A). In contrast, the activity of U-937/CR cell extracts was inhibited only in part in the presence of 500 μM 9-NC (Fig. 5A). A change from valine (GTT) to alanine (GCT) at position 145; and (2) a change in sequence at codon 591 (ACG to ACA; both coding for threonine). More importantly, sequences in the parental and resistant cells differed by a change in phenylalanine (Val) to serine (TCC) in U-937/CR cells (Fig. 6). These findings were confirmed in experiments that utilized products from independent PCR reactions.

**Expression of the Mutant Topo I Gene in E. coli**—In order to determine whether the alteration in the topo I gene from U-937/CR cells confers CPT resistance, we expressed the mutant cDNA in E. coli and assayed for sensitivity to 9-NC. In order to eliminate potential confounding effects of E. coli topoisomerases, we expressed human topo I as a fusion protein linked to GST and partially purified the enzyme from bacterial lysates using glutathione Sepharose beads. Topo I catalytic activity in the purified bacterial lysates was also assayed in the absence of Mg2⁺ (1). Under these conditions, there was no detectable catalytic activity in purified lysates from bacteria expressing GST alone (data not shown). In contrast, purified lysates from bacteria expressing a GST-topo I fusion protein exhibited topo I catalytic activity that was inhibited by 50 μM 9-NC (Fig. 7). Moreover, purified lysates from bacteria expressing a fusion protein containing the topo I mutation at position 361 exhibited catalytic activity that was unaffected by 50 or 100 μM of 9-NC (Fig. 7). These findings indicated that substitution of phenylalanine with serine at position 361 of human topo I is sufficient to confer resistance to the inhibitory effects of 9-NC on catalytic activity.

**DISCUSSION**

Previous work has demonstrated that cellular resistance to CPT is associated with decreases in topo I activity. Selection of P388 cells resistant to CPT has been associated with decreases (2-4-fold) in topo I mRNA, immunoreactivity, and extractable enzymatic activity (23). This induction of CPT resistance has also been associated with rearrangements and increases in methylation of the topo I gene (23, 27). Other studies in human tumor cell lines have shown that resistance to CPT and its derivative CPT-11 is associated with a reduction, but not qualitative changes, in topo I protein (26). Selection of human lung cancer cells for resistance to CPT-11 has been associated with a

**TABLE II**

| Drug | IC₅₀ | U-937 | CR |
|------|-----|-------|----|
| 9-NC | 30 ± 2.56 μM | 684 ± 6.83 μM |
| 9-NC + verapamilb,c | 31 ± 0.25 μM | 601 ± 59.2 μM |
| CPT | 0.42 ± 0.018 μM | 7.0 ± 0.32 μM |

* Expressed as mean ± standard error.

* Verapamil concentration 10 μg/ml.

* The CR values for 9-NC with or without verapamil are not statistically different (p = 0.277, paired two-tailed t test).
U-937/CR cells. A, Increasing amounts of nuclear extracts for 30 min at 37 °C. Relaxed DNA amounts of nuclear extract were:

was scanned with a laser densitometer to quantitate catalytic activity.

Similar results were obtained in two separate experiments.

There was also no detectable change in protein levels associated with acquisition of 9-NC resistance. The results have been obtained in V79 hamster lung cells (24), while another CPT-resistant line derived from hamster lung exhibited decreased catalytic activity and no change in immunoactive top0 I protein (29). These findings are thus in contrast to previous CPT-resistant isolates which have associated decreases in top0 I expression.

Immunoblot analysis of U-937/CR extracts with an anti-top0 I antibody revealed reactive proteins at 100, 75, and 67 kDa. While previous studies have demonstrated that the 100- and 67-kDa proteins are catalytically active (43, 49), less is known about the 75-kDa species (50). However, the finding that the pattern and intensity of the three bands was similar in both U-937 and

U-937/CR cells were found to have increased levels of top0 I1.

![Figure 4](image_url)

**Figure 4.** Topo I catalytic activity is similar in U-937 and U-937/CR cells. A, Supercoiled plasmid DNA was incubated with decreasing amounts of nuclear extracts for 30 min at 37 °C. Relaxed DNA products were detected by electrophoresis in a 0.8% agarose gel containing 2 μg/ml chloroquine. SC represents supercoiled DNA. The amounts of nuclear extract were: lane 1, no nuclear extract; lanes 2 and 7, 200 ng; lanes 3 and 8, 67 ng; lanes 4 and 9, 33 ng; lanes 5 and 10, 17 ng; lanes 6 and 11, 8 ng. B, the photographic negative of the above gel was scanned with a laser densitometer to quantitate catalytic activity in U-937 (A) and U-937/CR (C) cells. Intensity of the bands corresponding to supercoiled DNA was compared to that for the band in lane 1. Similar results were obtained in two separate experiments.

![Figure 5](image_url)

**Figure 5.** Effects of 9-NC on topo I activity of nuclear extracts from U-937 and U-937/CR cells. Supercoiled plasmid DNA was incubated with 150 ng of nuclear extract and increasing concentrations of 9-NC for 30 min at 37 °C. Relaxed DNA was detected as described in the legend to Fig. 4. A, lane 1 represents a reaction performed in the presence of 500 μM 9-NC and no nuclear extract. The concentrations of 9-NC in the remaining lanes were: lanes 2 and 8, 0 μM; lane 3, 1 μM; lanes 4 and 9, 5 μM; lanes 5 and 10, 50 μM; lanes 6 and 11, 100 μM; lanes 7 and 12, 250 μM; lane 13, 500 μM. B, quantitation of supercoiled bands for U-937 (A) and U-937/CR (C) cells as described in the legend to Fig. 4. Similar results were obtained in two separate experiments.

![Figure 6](image_url)

**Figure 6.** Identification of a mutation at codon 361 in U-937/CR cells. Reverse transcription and PCR were used to isolate topo I cDNAs from U-937 and U-937/CR cells (primers listed in Table I). The products were directly sequenced using the dideoxynucleotide method.

Changes, this loss of 9-NC sensitivity probably accounts for the comparable increases in cellular resistance to this agent. Immunoblot analysis of U-937/CR extracts with an anti-topo I antibody revealed reactive proteins at 100, 75, and 67 kDa. While previous studies have demonstrated that the 100- and 67-kDa proteins are catalytically active (43, 49), less is known about the 75-kDa species (50). However, the finding that the pattern and intensity of the three bands was similar in both U-937 and
U937/CR cells further supported the absence of quantitative alterations to account for resistance. In contrast, analysis of top1 DNA demonstrated identical sequences in U937 and U937/CR cells with the exception of a mutation in codon 361 that results in the substitution of phenylalanine with serine. Moreover, expression of a wild-type allele was undetectable in U937/CR cells. As determined by Southern analyses, this finding was not related to genomic rearrangements (data not shown). Although we were able to detect differences in methylation of the top1 gene in the resistant cells (data not shown), the basis for lack of expression of a wild-type allele is unclear. While these findings, taken together, supported the involvement of a mutation at codon 361 in cellular resistance to 9-NC, a more direct analysis was performed using partially purified human top1 expressed in E. coli. The results of these experiments demonstrate that expression of top1 containing the observed mutation at codon 361 is sufficient for conferring resistance to 9-NC.

Comparison of amino acids 352 to 371 in human top1 with corresponding sequences of this enzyme from other species has revealed a region that is highly conserved in eukaryotes (Table III). Moreover, vaccinia virus top1, which is resistant to CPT, has a peptide sequence that differs in this region. Thus, one potential explanation for the present findings is that this highly conserved domain in eukaryotes is involved in the binding of 9-NC to the top1-DNA complex. A mutation at codon 361 could directly interfere with 9-NC binding. Alternatively, an alteration at this site could influence secondary structure and thereby binding of 9-NC. In this context, use of a computer algorithm to predict protein secondary structure and thereby binding of 9-NC is in progress.

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