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Efficacy of Trimethoprim in Murine Experimental Infection with a Thymidine Kinase-Deficient Mutant of *Escherichia coli*

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The antimicrobial activity of trimethoprim is antagonized by thymidine in in vitro susceptibility tests. The purpose of this investigation was to determine whether this antagonism also occurred during experimental infection in mice, which have high serum thymidine concentrations. We derived a mutant strain of *Escherichia coli*, TT-48, incapable of utilizing exogenous thymidine from parent strain *E. coli* KC-14 and then investigated the in vitro and in vivo antimicrobial activities of trimethoprim, sulfamethoxazole, cefdinir, and ofloxacin against these strains. *E. coli* TT-48 lacked the activity of thymidine kinase, which catalyzes the conversion of thymidine to thymidylate, but its growth curve remained close to that of the parent strain. The MICs of all of the antimicrobial agents tested, except cefdinir, for the mutant strain were slightly inferior to those for the parent strain. The bactericidal effect of trimethoprim against the parent strain was antagonized by thymidine at concentrations of more than 1 μg/ml, while that against the mutant strain was not affected by thymidine even at the highest concentration (10 μg/ml). The therapeutic efficacy of trimethoprim in experimental murine infections was significantly higher when the mutant rather than the parent strain was used, whereas the therapeutic efficacy of cefdinir or ofloxacin, whose antimicrobial action is independent of folic acid synthesis, was the same with both strains. Unexpectedly, sulfamethoxazole also had similar efficacy against both strains. Thus, high thymidine concentrations antagonized the antimicrobial activity of trimethoprim in vitro and in vivo.

Trimethoprim, which has been used clinically either alone or in combination with a sulfonamide (e.g., sulfamethoxazole, sulfadiazine, sulfamoxole), is a synthetic, broad-spectrum antimicrobial agent which acts as an inhibitor of bacterial dihydrofolate reductase (9). The major consequence of this inhibition is a drastic and rapid reduction in the levels of tetrahydrofolate and its cofactors, which are required by the bacterial cell for the synthesis of purines, pyrimidines, and certain amino acids (3, 10).

It has been known for many years that the in vitro antimicrobial activity of trimethoprim against various organisms is inhibited by the thymidine contained in commercially prepared media (12, 19). Then and Angehrn (22) reported that the antimicrobial activity of trimethoprim against *Escherichia coli* B and *E. coli* 15 in human blood and urine was inhibited by supplementing the medium with thymidine. Hamilton-Miller (7) also reported that the antimicrobial activity of trimethoprim against different species of gram-positive cocci (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, etc.) was antagonized by thymidine added to Iso-Sensitest medium that did not contain any thymidine or thymine.

Then (21) argued that testing dihydrofolate reductase inhibitors in mice or rats always resulted in very high 50% effective doses (ED_{50}s). Nottebrock and Then (16) also reported that considerable amounts of thymidine were present in murine serum and urine (163 to 166 and 410 to 728 ng/ml, respectively) and that little or no thymidine is usually found in human serum and urine. This led us to hypothesize that the considerable amounts of thymidine present in murine serum may interfere with the in vivo activity of trimethoprim.

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The present investigation was performed to determine the effect of thymidine on the antimicrobial action of trimethoprim in murine systemic infection. From parent strain KC-14, we derived a mutant strain of *E. coli* (TT-48) which was incapable of incorporating thymidine. Both the parent and the mutant strains possessed strong virulence toward mice. We furthermore showed that thymidine is a potential inhibitor of the antimicrobial activity of trimethoprim in vivo as well as in vitro.

MATERIALS AND METHODS

Bacterial strain. *E. coli* KC-14, which has murine virulence, was used as a stock culture from the Department of Microbiology, Kyoto Pharmaceutical University.

Antimicrobial agents. The antimicrobial agents used in the present study were obtained as standard laboratory powders and were used immediately after dilution. The agents and their sources were as follows: trimethoprim and sulfamethoxazole (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan), ofloxacin (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), and cefdinir (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan).

Media. Heart infusion agar (HIA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and Iso-Sensitest broth (Oxoid, Basingstoke, United Kingdom), which does not contain thymidine or thymine, were used. Peptone-glucose (2% polypeptone, 0.5% NaCl, and 0.2% glucose; pH 7.2) medium was used to screen for *E. coli* TT-48.

Mice. Four-week-old male ddY mice were purchased from Japan SLC Co. (Shizuoka, Japan).

Mutant isolation. The method described by Hiraga et al. (8) was used with minor modification. Mutagenesis was effected by using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) at 50 μg/ml. Mutagenized cells were grown in HIA; replicated on peptone-glucose medium containing 25 μg of 5-fluoro-2′-deoxyuridine (Sigma), 25 μg of uridine (Sigma), and 50 μg of thymidine (Wako Pure Chemical Co., Ltd., Osaka, Japan) per ml; and incubated at 37°C for 20 h. 5-Fluoro-2′-deoxyuridine is a well-known inhibitor of DNA synthesis (by inhibition of thymydilate synthetase, which synthesizes endogenous thymidylate) and RNA synthesis in *E. coli*, and these effects can be overcome only when excess uridine and thymidine are added to the medium (24). A colony which did not grow on this medium was selected as a mutant strain unable to utilize exogenous thymidine (*E. coli* TT-48) and was used in the following experiments.

Incorporation of [*methyl*-^{3}H]thymidine into DNA. The method described by Boyce and Setlow (2) was used with minor modification. Cells were grown to exponential phase in M9 minimal medium supplemented with 0.2% Casamino

Acids and 0.025% deoxyadenosine. Following the addition of 26.5 nM [*methyl*-^{3}H]thymidine (specific activity, 5 Ci/mmol; Amersham Canada Ltd., Oakville, Canada), the mixture was incubated at 37°C with shaking. At various times, 200-μl aliquots were taken and added to 200 μl of cold 10% trichloroacetic acid (TCA). After 30 min of extraction on ice, acid-precipitable material was collected on 0.45-μm-pore-size membrane filters (Advantec, Tokyo, Japan); this material was washed with 10 ml of cold 5% TCA and then with 5 ml of water. The membranes were dried and immersed in scintillation fluor (Amersham), and radioactivity was counted in a Liquid Scintillation Analyzer 2000 CA (Packard Instrument Co. Inc., Meriden, Conn.).

Enzyme preparation. Cells were suspended with 70 mM Tris-HCl buffer (pH 7.8) and disrupted with a W-225R cell disruptor (Heat System-Ultrasonic Inc., Plainview, N.J.) in ice-water. After centrifugation for 30 min at 10,000 × *g* at 4°C, the resulting supernatant fluid was used as a crude extract. The amount of protein contained in the extract was measured by the method of Lowry et al. (14).

Deoxythymidine kinase assay. Thymidine kinase activity was assayed by the method described by Furlong (5). In brief, the reaction mixture (final volume, 100 μl) contained 75 μl of the stock assay solution (10 μM $MgCl_2$, 20 μM NaF, 10 μM ATP, 0.03% bovine serum albumin, and 600 nM [*methyl*-^{3}H]thymidine in 70 mM Tris-HCl [pH 7.8]) and 25 μl of the crude extract. This mixture was incubated at 37°C. At various times during incubation, assay tubes were taken out, heated for 5 min in a boiling-water bath to stop the reaction, chilled in ice-water, and centrifuged at 10,000 × *g* for 5 min. Two 20-μl aliquots of the supernatant were transferred to duplicate sets of DEAE disks (DE 81, 2.4-cm diameter; Whatman Co., Ltd., Tokyo, Japan). One set of disks was washed three times with 95% ethanol at room temperature, allowing approximately 10 ml of ethanol for each disk and 5 min of intermittent swirling for each wash. After washing, phosphorylated [*methyl*-^{3}H]thymidine was adsorbed to the DEAE disk and nonreacted [*methyl*-^{3}H]thymidine was not. These disks were then dried. The second set of disks was dried on a nonwettable surface without washing. All disks were immersed in scintillation fluor, and radioactivity was counted with a scintillation counter as described previously. Percent conversion at each time point was calculated from the ratio between washed and unwashed disks.

Thymidine phosphorylase assay. The activity of thymidine phosphorylase was assayed by the method described by Krenitsky et al. (13). The reaction mixture, containing 10 mM thymidine, 0.1 M sodium phosphate buffer (pH 7.4), and crude extract, was incubated at 37°C. At various times, 0.2-ml aliquots were removed, added to 0.2 ml of 1 M NaOH, and diluted to 2 ml with distilled water. The increase in absorbance at 300 nm of the reaction product, thymine, was measured by the use of a UV-visisible spectrum recording spectrophotometer (UV-2200; Shimadzu Co. Ltd., Kyoto, Japan).

Killing kinetics. An 18-h culture of *E. coli* was 100-fold diluted, and 50 μl of this bacterial suspension was inoculated into 5 ml of Iso-Sensitest broth (Oxoid) and cultured with shaking. After 2.5 h, trimethoprim or sulfamethoxazole at a final concentration of four times the MIC and thymidine at final concentrations of 0, 0.1, 1, and 10 μg/ml were simultaneously added to cultures. Quantitative subculturing was performed at 0, 1, 2, 3, and 4 h of incubation by plating 0.1-ml specimens onto HIA plates after serial 10-fold dilution. No steps were taken to exclude any potential carryover effect, as both trimethoprim and sulfamethoxazole at below half their MICs exhibited negligible activity against both strains.

MIC determinations. MICs were determined by the agar dilution method recommended by the Japanese Society of Chemotherapy (4). An inoculum of 10^4 CFU was spotted onto the antimicrobial agent-containing Iso-Sensitest agar by using a multiple inoculator (Microplanter; Sakuma Seisakusyo, Tokyo, Japan). All plates were incubated at 37°C for 18 h. The MIC was defined as the lowest concentration of drug that prevented visible growth.

In vivo activities. The in vivo activities of the drugs were determined against systemic infections in mice. Test organisms for infections were cultured on nutrient agar (Nissui) at 37°C for 18 h and were suspended in 3% gastric mucin (Wako) for systemic infections. Mice (weight, 18 to 20 g) in groups of 10 mice each were inoculated intraperitoneally with 0.5 ml of a bacterial suspension corresponding to an inoculum ranging from 10 to 100 times the 50% lethal dose (LD_{50}) of bacteria. Two hours after infection, mice were orally given various doses of trimethoprim, sulfamethoxazole, ofloxacin, or cefdinir ranging from 0.156 to 400 mg/kg of body weight. The total number of mice surviving at each dose level was recorded on day 7 after infection, and the ED_{50} was calculated by the method of probit (1). All untreated mice died within 2 days after infection.

RESULTS

Incorporation of [*methyl*-^{3}H]thymidine. The mutant *E. coli* strain (TT-48) was derived from the parent strain (KC-14) after mutagenesis and selection for inability to utilize exogenous thymidine. Figure 1 shows the growth properties of and thymidine incorporation by the parent and mutant *E. coli* strains. The growth curve of *E. coli* TT-48 was close to that of *E. coli* KC-14. Microscopic examination revealed that the state of cell division and the cellular morphology were the same in both strains (data not shown). However, *E. coli* TT-48, in contrast to *E. coli* KC-14, failed to incorporate appreciable amounts of [*methyl*-^{3}H]thymidine into the TCA-insoluble fraction: this showed that *E. coli* TT-48 could not incorporate exogenous thymidine into its DNA.

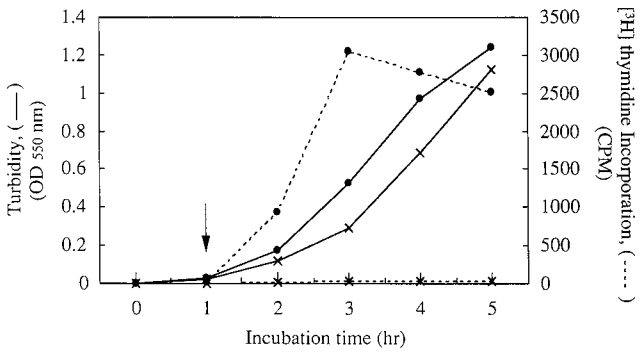

FIG. 1. Growth and incorporation of [*methyl*-^{3}H]thymidine in *E. coli* KC-14 (●) and TT-48 (×). The arrow indicates the time at which [*methyl*-^{3}H]thymidine was added.

Activities of thymidine phosphorylase and thymidine kinase. The activities of thymidine phosphorylase and thymidine kinase were determined in crude extracts for the parent and mutant strains (Fig. 2). Both strains showed a linear increase in the accumulation of product (thymine) with incubation time (Fig. 2A). However, only the parent strain showed any significant adsorption of phosphorylated [*methyl*-^{3}H]thymidine (Fig. 2B).

In vitro antimicrobial activity. The MICs of trimethoprim and reference drugs, i.e., sulfamethoxazole, ofloxacin, and cefdinir, against the two strains are summarized in Table 1. The susceptibility of *E. coli* TT-48 to each antimicrobial agent, except cefdinir, was slightly lower than that of *E. coli* KC-14.

Effect of thymidine on killing kinetics of trimethoprim and sulfamethoxazole. The results of this experiment are presented in Fig. 3. Both strains were inhibited in a bacteriostatic way at the MIC of trimethoprim (data not shown) and in a bactericidal way at four times the MIC of trimethoprim. However, the bactericidal effect of trimethoprim against *E. coli* KC-14 was entirely antagonized at thymidine concentrations of more than 1 μg/ml. In contrast, *E. coli* TT-48 was killed by trimethoprim regardless of the thymidine concentration. The same result occurred with sulfamethoxazole (data not shown).

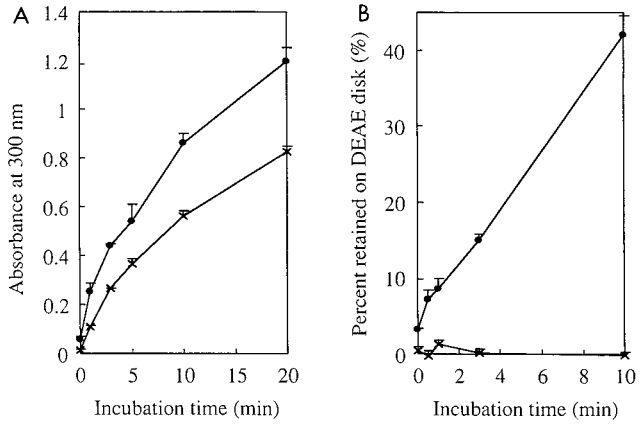

FIG. 2. Activities of thymidine phosphorylase (A) and thymidine kinase (B) in extracts of *E. coli* KC-14 (●) and TT-48 (×).

TABLE 1. Effects of trimethoprim and reference drugs against systemic murine infections caused by *E. coli* KC-14 and TT-48

E. coli strain	Infective dose (CFU/mouse) (LD_{50} equivalent)	Drug	MIC (μg/ml)	ED_{50} (mg/kg) (95% confidence limits)
EC-14	5.9×10^5 ($77 \times KD_{50}$)	Trimethoprim	0.025	196.45 (110.71–499.00)
		Sulfamethoxazole	3.13	12.30 (4.08–114.29)
		Ofloxacin	0.013	0.09 (0.02–1.39)
		Cefdinir	0.10	2.04 (0.77–32.09)
TT-48	4.6×10^5 ($18 \times LD_{50}$)	Trimethoprim	0.05	8.57 (5.26–13.42)
		Sulfamethoxazole	6.25	12.37 (7.92–20.85)
		Ofloxacin	0.025	0.27 (0.20–0.36)
		Cefdinir	0.10	2.64 (1.74–3.85)

Antimicrobial agent efficacy in murine systemic infection with *E. coli* KC-14 and TT-48. The therapeutic efficacies of each antimicrobial agent against both strains are shown in Table 1. The in vitro antimicrobial activity of trimethoprim against *E. coli* KC-14 was superior to that of sulfamethoxazole, but in experimental infection with *E. coli* KC-14, the therapeutic efficacy of trimethoprim was extremely inferior to that of sulfamethoxazole. However, during murine infection with *E. coli* TT-48, the therapeutic effect of trimethoprim was remarkably improved. The ED_{50} of trimethoprim against *E. coli* TT-48 was approximately 1/23 of that against *E. coli* KC-14. On the other hand, the ED_{50}s of sulfamethoxazole against the two strains were almost equal. The therapeutic efficacy of ofloxacin against *E. coli* TT-48 was inferior to that against *E. coli* KC-14. Cefdinir showed similar ED_{50}s in both strains.

DISCUSSION

In the present study, we used *E. coli* KC-14 to derive a mutant strain (*E. coli* TT-48) which was incapable of utilizing exogenous thymidine. In murine systemic infection with *E. coli* KC-14, trimethoprim did not show high therapeutic efficacy despite showing high antimicrobial activity during in vitro susceptibility testing, while trimethoprim showed high therapeutic efficacy in murine systemic infection with the mutant strain TT-48.

Then and Angehrn (22) indicated that the addition of thymidine to M9 minimal salts medium dose-dependently inhibits the antimicrobial activity of trimethoprim against *E. coli* B. In *E. coli*, exogenous thymidine is converted by thymidine kinase to thymidylate (salvage pathway), which is one of the precursors of DNA structural elements (11). In addition, *E. coli* possesses another pathway for synthesis of thymidylate; the key enzyme of this pathway is thymidylate synthetase, which requires 5,10-methylene-tetrahydrofolate and converts dUMP to thymidylate. Hiraga et al. (8) reported that a mutant thymidine kinase-deficient strain of *E. coli* was incapable of growth even in medium containing thymidine and uridine when thymidylate synthetase was inhibited by 5-fluoro-2′-deoxyuridine. On the other hand, the parent strain was able to grow in the same conditions. Trimethoprim acts as an inhibitor of bacterial dihydrofolate reductase (9) to inhibit the synthesis of endogenous thymidylate. From these reports, it is reasonable to speculate that trimethoprim could not inhibit the supply of thymidylate by thymidine kinase, so that if a considerable amount of thymidine is present in the growth medium, the parent strain is able to grow even in the presence of trimethoprim.

The mutant strain *E. coli* TT-48 could not incorporate exogenous thymidine into DNA (Fig. 1), and thymidine kinase activity disappeared entirely (Fig. 2). It was clear that the mutation of *E. coli* TT-48 was related to a lack of thymidine kinase activity. When the MIC of each antimicrobial agent was measured with Iso-Sensitest medium (which does not contain thymidine or thymine), the sensitivity of mutant strain *E. coli* TT-48 was slightly inferior to that of parent strain *E. coli* KC-14 (Table 1). For both strains, trimethoprim was inhibited in a bactericidal way at four times the MIC in the medium (Fig. 3). However, the bactericidal effect on *E. coli* TT-48, unlike that on *E. coli* KC-14, was not antagonized by the addition of thymidine. This difference appears to be related to a deficiency of the thymidine kinase-dependent salvage pathway in *E. coli* TT-48. The data given above suggest that the in vitro antimicrobial activity of trimethoprim is influenced by the ability of *E. coli* KC-14 to incorporate exogenous thymidine.

We evaluated the in vivo activity of trimethoprim against murine experimental infections. In in vivo experimental infection with *E. coli* KC-14, the ED_{50} of trimethoprim was markedly higher than that which would have been expected from in vitro susceptibility testing. Nottebrock and Then (16) previously reported that the thymidine concentration in murine serum reaches 166 ng/ml, while that in human serum is less than 10 ng/ml. In addition, they noted that the thymidine concentration in murine serum increased further during experimental bacterial infection. Then (21) also suspected that the large amounts of thymidine in murine plasma might interfere with the in vivo antimicrobial activity of trimethoprim. Therefore, by using the mutant strain *E. coli* TT-48, we attempted to prove that the bactericidal action of trimethoprim was inhibited by murine body fluids containing considerable amounts of thymidine. In experimental infection with *E. coli* TT-48, only the therapeutic efficacy of trimethoprim was markedly improved compared with the efficacies of reference drugs. In addition, the in vitro antimicrobial activity of trimethoprim

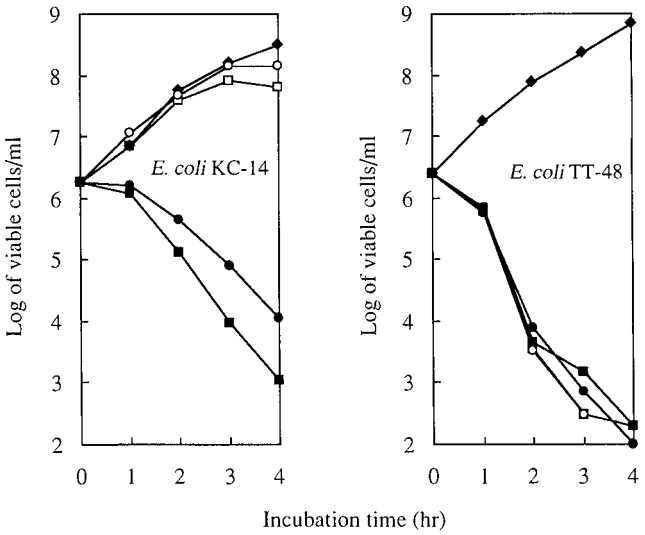

FIG. 3. Antimicrobial effect of trimethoprim (four times the MIC) on *E. coli* strains and antagonism of it by various concentrations of thymidine. Symbols indicate thymidine concentrations: ◆, control; ■, no thymidine; ●, 0.1 μg/ml; □, 1 μg/ml; ○, 10 μg/ml.

against *E. coli* TT-48 was not antagonized by the addition of thymidine (Fig. 3). The serum thymidine levels of uninfected ddY mice were approximately 340 ng/ml in a high-performance liquid chromatography assay (data not shown). It was speculated that serum thymidine concentrations in infected ddY mice were extremely high and were sufficient to inhibit the antimicrobial activity of trimethoprim against *E. coli* KC-14. These results indicate that the antimicrobial activity of trimethoprim in murine systemic infection with *E. coli* KC-14 is strongly inhibited by thymidine in serum. In Fig. 3, the bactericidal effect of trimethoprim against *E. coli* TT-48 was a little greater than that against *E. coli* KC-14. We do not consider that this significantly affects the ED_{50}, because the concentration of trimethoprim in serum is higher than the MICs of the drug for both strains until 4 h at least (data not shown).

In separate experiments (data not shown), we investigated the in vitro relationship between the antimicrobial effect of trimethoprim and thymidine in *S. aureus* and found that the antimicrobial activity of trimethoprim was entirely antagonized by more than 1 μg of thymidine per ml. It is speculated that other bacteria that take up exogenous thymidine are also able to escape the antimicrobial action of trimethoprim in mice.

There was no difference in ED_{50}s of cefdinir for the parent and mutant strains of *E. coli*, which is not surprising, considering that the mechanism of antimicrobial action of cefdinir is unlikely to be affected by thymidine (23). Similarly, for ofloxacin, there was little difference in ED_{50}s for the two strains, which is again not unexpected, considering that the mechanism of antimicrobial action of ofloxacin is inhibition of DNA gyrase activity (20) and is therefore unlikely to be affected by thymidine metabolism. The ED_{50}s of sulfamethoxazole against the two strains were similar. Sulfamethoxazole acts as an inhibitor of bacterial dihydropteroate synthetase and inhibits folate synthesis. Like trimethoprim, the in vitro antimicrobial activity of sulfamethoxazole was inhibited by thymidine in *E. coli* KC-14 but not in *E. coli* TT-48 (data not shown). One reason for this difference may be the pharmacokinetics of sulfamethoxazole (17). When administered orally, levels of sulfamethoxazole in plasma are higher and more prolonged than those of trimethoprim (15, 18). Another reason may be that sulfamethoxazole acts by activating phagocytes in murine mesentery (6). The therapeutic efficacy of sulfamethoxazole might therefore escape any influence from thymidine.

In conclusion, we have demonstrated that the in vivo antimicrobial activity of trimethoprim is inhibited by thymidine in a manner similar to that seen in vitro. For this reason, we do not believe it is appropriate to use animal species with high thymidine concentrations in body fluids when assessing the likely clinical efficacy of trimethoprim or its analogs (e.g., brodimoprim).

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