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Combined therapy with amphotericin B and caspofungin in an experimental model of disseminated histoplasmosis

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ABSTRACT

Objective. To assess the effect of amphotericin B and caspofungin, as well as their combinations in the therapy of experimental disseminated histoplasmosis. **Material and methods.** BALB/c mice were intraperitoneally infected with four different strains of *Histoplasma capsulatum* and given to antifungal treatments. The response to intraperitoneal therapy with amphotericin B (0.5, 1.0, and 2.0 mg/kg of body weight) or caspofungin (10 mg/kg of body weight) and their combinations, was evaluated by the quantification of yeast colony-forming units (CFU) per gram of spleen or lung, from each animal. Additionally, the pathogen was monitored histopathologically in the excised organs. Data were analyzed with the Kruskall-Wallis and Tukey tests. **Results.** Caspofungin was more effective than amphotericin B in reducing the CFU/g. A synergistic effect was observed when caspofungin (10 mg/kg) was combined with amphotericin B (0.5 or 1.0 mg/kg). Significant differences in CFU values, H = 119.78 (P = 0.00001), were found among the treatment groups. However, statistical analyses did not reveal significant differences, H = 2.837 (P = 0.428), in the therapeutic responses with the four *H. capsulatum* strains tested. **Conclusion.** Combined therapy with amphotericin B and caspofungin could represent an alternative treatment to be explored in severe human histoplasmosis.

Key words. Antifungal drugs. Combined therapy. Amphotericin B. Caspofungin. Histoplasmosis.

Terapia combinada con anfotericina B y caspofungina en un modelo experimental de histoplasmosis diseminada

RESUMEN

Objetivo. *Evaluar el efecto de la anfotericina B y la caspofungina, así como de sus combinaciones en la terapia de la histoplasmosis diseminada experimental.* ***Material y métodos.*** *Ratones BALB/c fueron infectados intraperitonealmente con cuatro diferentes cepas de* Histoplasma capsulatum *y sujetos a tratamientos antifúngicos. La respuesta a la terapia intraperitoneal con anfotericina B (0.5, 1.0 y 2.0 mg/kg de peso) o caspofungina (10 mg/kg de peso) así como con sus combinaciones, se evaluó mediante la cuantificación de levaduras a través de unidades formadoras de colonias (UFC) por gramo de bazo y pulmón de cada animal. Adicionalmente, el patógeno se rastreó histopatológicamente en los órganos extraídos. Los datos se analizaron mediante las pruebas de Kruskall-Wallis y Tukey.* ***Resultados.*** *La caspofungina fue más efectiva en la reducción de las UFC/g que la anfotericina B. Se observó un efecto sinérgico cuando se combinaron la caspofungina (10 mg/kg) con la anfotericina B (0.5 o 1.0 mg/kg). Se encontraron diferencias significativas en los valores de UFC, H = 119.78 (P = 0.00001), entre los grupos bajo tratamiento. Sin embargo, el análisis estadístico no reveló diferencias significativas en la respuesta terapéutica, H = 2.837 (P = 0.428), con las cuatro cepas de* H. capsulatum *probadas.* ***Conclusión.*** *La terapia combinada de anfotericina B con caspofungina podría representar un tratamiento alternativo a explorar en la histoplasmosis humana severa.*

Palabras clave. *Drogas anti-fúngicas. Terapia combinada. Anfotericina B. Caspofungina. Histoplasmosis.*

INTRODUCTION

Histoplasma capsulatum var. *capsulatum* is the etiologic agent of the systemic mycosis "histoplasmosis capsulati". It is a saprobe and dimorphic fungus that grows in a mycelial phase in nature (infective form, at 25 ºC), and in a parasitic yeast phase (virulent form, at 37 ºC) when it infects susceptible hosts. This pathogen is distributed worldwide, particularly in tropical and subtropical areas.[1] In contrast to other American countries, primary pulmonary histoplasmosis (PPH) is the most important clinical form in Mexico. Although cutaneous and mucocutaneous manifestations of disseminated histoplasmosis have been reported in Mexican AIDS-histoplasmosis patients, PPH associated with an epidemic form of the disease still impacts the rural and urban areas of the country.[2-6] In general, PPH has a benign course and presents a variety of clinical manifestations ranging from mild to severe, depending on the number of inhaled propagules and the immune condition of the infected individual. Mexico has the highest PPH-fatality rate in the world due to frequent outbreaks and PPH is considered an occupational health issue, further highlighting its relevance in the country.[2 3 6-10]

Although anti-retroviral therapy has decreased AIDS-associated histoplasmosis worldwide, this connection prevails in many countries. Overall, acute disseminated histoplasmosis is seen mainly in immunocompromised patients with different etiologies.[11-13] Even with an inherent bias due to the under-reporting of histoplasmosis cases, as well as the lack of official information detailing the incidence of histoplasmosis in Mexico, recent data suggest an important increase in the mortality due to the disease. This is despite the availability of treatments against the severe clinical forms and is probably the result of the misdiagnosis of histoplasmosis, or the presence of histoplasmosis refractory to the usual antifungal agents.

The treatment of most histoplasmosis patients primarily involves amphotericin B monotherapy. However, given that amphotericin B treatment requires strict medical surveillance, due to the need for long periods of administration and its nephrotoxicity, as well as the high cost of the treatment, other therapies are often necessary. Other antifungals used for the treatment of histoplasmosis include the triazole family, such as itraconazole and fluconazole.[14] Although echinocandins have not been used as optional therapy in histoplasmosis, they could be considered as substitute drugs in refractory histoplasmosis, taking into account their successful results in amphotericin B-refractory aspergillosis.[15 16] The present study tested the combined therapy of amphotericin B and caspofungin (echinocandin), as well as monotherapy with either drug in a murine model of disseminated histoplasmosis. This strategy is based on the use of two antifungal agents with different molecular targets in order to develop alternative treatment regimens for *H. capsulatum* infection.

MATERIAL AND METHODS

Strains

The yeast phase of four *H. capsulatum* strains (EH-53, EH-359, H.1.07.W, and G-186B) was used. Strains EH-53 and EH-359 were derived from Mexican patients with disseminated histoplasmosis and strain H.1.07.W was isolated from a Guatemalan patient with AIDS-associated histoplasmosis. Strain G-186B (ATCC 26030) was used as reference. All strains belong to the *Histoplasma capsulatum* Strain Collection of the Fungal Immunology Laboratory of the Departamento de Microbiologia-Parasitologia, Facultad de Medicina, Universidad Nacional Autonoma de Mexico (UNAM), which is registered in the World Data Centre for Microorganisms (WDCM) database with the acronym LIH-UNAM WDCM817. Information on strains is available at the website: http://histoplas-mex.unam.mx. Yeasts were maintained at 37 ºC in brain-heart-infusion medium (BHI) (Bioxon, Becton-Dickinson, Mexico City) supplemented with 0.1% L-cysteine and 1% glucose.

Mice

Four-week-old male syngeneic BALB/c mice of similar weights were provided by the animal housing facilities of the Facultad de Medicina, UNAM. Mice were kept under optimal environmental conditions and fed *ad libitum* with Purina (Purina de Mexico, Mexico City) and acidified distilled water. Mice were maintained and manipulated according to the Ethical Committee of the Facultad de Medicina, UNAM.

Infection

Suspensions of each *H. capsulatum* strain, containing 2 x 10^6 yeasts/mL, were prepared in isotonic saline solution (SS) and adjusted to two optical density (OD) units. The yeast suspension (0.2 mL) was inoculated by intraperitoneal route into each mouse. The selected fungal strains, infection dose, inocula-

tion route, and animal model have been previously optimized in our laboratory and have been shown to induce disseminated histoplasmosis.[17]

Therapeutic assessment of amphotericin B, caspofungin, and their combinations in experimental disseminated histoplasmosis

Seven groups, with four infected mice each, were used for every assay. An additional group was injected with SS, as negative control. Intraperitoneal antifungal therapy began 48 h after infection and continued for six days, according to the schedule shown in table 1. Three mice from each group were killed after the therapeutic treatment and their spleen and lungs were extracted to quantify the number of viable yeast present in the infected tissue, whereas the fourth animal was used only for histopathologic procedures.

Response to therapy was determined by quantifying the viable yeasts through the colony-forming units (CFU) per gram of organ (spleen or lung) from each animal.

Clinical signs of disease (weight loss, hirsute hair, hunched posture, immobilization, segregation, and death) were also recorded.

Determination of the CFU

Spleen and lungs were processed independently. They were weighed and homogenized in 150 mM PBS, pH 7.2, under strict sterile conditions. Each organ homogenate was PBS-diluted and 100 µL of each dilution was plated, in duplicate, on BHI-agar supplemented with *H. capsulatum* growth factor.[18] After two to seven days of incubation at 37 ºC, the CFU/g was quantified in each tested organ. Therapies were considered effective when the number of CFU/g in drug-treated mice was fewer than the CFU/g detected in the untreated infected controls (group 1, Table 1).

Therapeutic effects of amphotericin B, caspofungin, and their combinations on the experimental histoplasmosis were first assessed with reference strain G-186B, using four independent assays. Afterwards, the other strains were tested. Taking into account the eight tested groups, each strain assay was performed with 96 mice.

Histopathological observations were performed to confirm the presence of the pathogen in the organs of the fourth mouse of each group (n = 32), using peryodic acid Schiff (PAS) and haematoxylin/eosin (HE) stains.

Table 1. Therapeutic schedule.

Groups	Treatments	Doses (mg/kg of weight)
1	SS	–
2	AmB	0.5 mg (every 48 h)
3	AmB	1 mg (every 48 h)
4	AmB	2 mg (every 48 h)
5	CAS	10 mg (every 12 h)
6	AmB + CAS	0.5 mg (every 48 h) + 10 mg (every 12 h)
7	AmB + CAS	1 mg (every 48 h) + 10 mg (every 12 h)
8	SS	–

Groups 1-7 were infected to produce murine disseminated histoplasmosis. Group 1 was used as an untreated infected control and group 8 as an uninfected control (see details under Material and methods). Forty-eight hours after infection, antifungal agents were administered intraperitoneally until the sixth day, according to the above schedule. AmB and CAS doses were based on previous references.[23,24] **A**m**B**: Amphotericin B. CA**S**: Caspofungin. SS: Saline solution. (–): Without therapy.

Statistical analyses

Data for therapies and strains tested were first analyzed by non-parametric Kruskal-Wallis variance analysis.[19] When significant differences among means were found, the data were analyzed using non-parametric Tukey's test for multiple comparisons.[19] Differences were considered statistically significant when P values were ≤ 0.05. The software OpenStat4 version 7.0 was used for statistical analyses.[20]

RESULTS

All infected mice developed the characteristic signs of murine histoplasmosis. When death occurred, mainly in the untreated infected controls (group 1, Table 1), animals were immediately processed. The successful response to the therapeutic schedule was confirmed by the disappearance of clinical signs. Uninfected controls were always healthy.

Therapeutic assessment of amphotericin B, caspofungin, and their combinations in experimental disseminated histoplasmosis

The average CFU/g values were determined with all mice from each group and figures 1 and 2 show representative data from four assays using the reference strain G-186B. Group 5, treated with caspofungin, showed very low CFU/g values in all the organs tested. Combined therapy with amphotericin B and caspofungin (groups 6 and 7) consistently yielded the

lowest CFU/g values (near zero), in both the spleen and lungs of infected mice (Figures 1 and 2). Results obtained with EH-53, EH-359, and H.1.07.W *H. capsulatum* strains were similar to those from the reference strain G-186B (data not shown).

In general, mice under treatment with amphotericin B (groups 2-4) showed a wide range of CFU/g values. The maximum value of CFU/g in spleen was 300 x 10^3 (group 2) and the minimum was 0.19 x 10^3 (group 4), whereas the maximum value in lungs was 370 x 10^3 (group 4) and the minimum was 0.36 x 10^3 (group 3). As expected, organs from untreated infected controls (group 1) had the highest CFU/g values (400 x 10^3). In addition, uninfected control animals (group 8) did not develop yeast colonies.

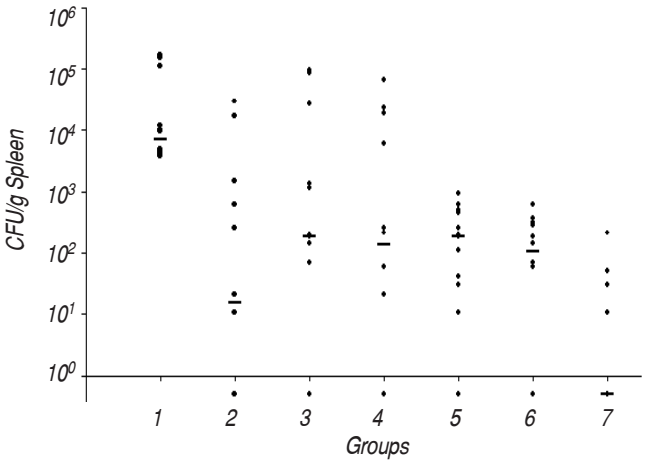

Figure 1. Effect of amphotericin B, caspofungin, and their combinations on CFU/g of spleen from mice infected with the reference strain G-186B. Group 1: infected controls; Groups 2-4: AmB 0.5, 1.0, and 2.0 mg/kg of weight, respectively; Group 5: CAS 10 mg/kg of weight; Group 6: AmB 0.5 mg/kg and CAS 10 mg/kg of weight; Group 7: AmB 1.0 mg/kg and CAS 10 mg/kg of weight. The uninfected control (Group 8) is not plotted. Four assays were performed using 12 mice for each therapeutic treatment group. **Bars:** *Median of each group.*

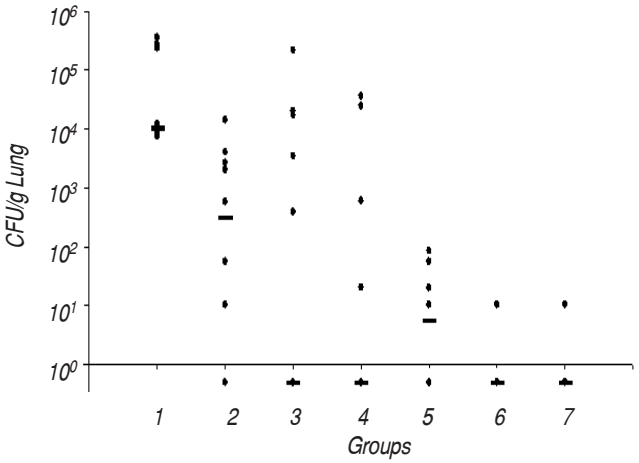

Figure 2. *Effect of amphotericin B, caspofungin and their combinations in CFU/g of lungs from mice infected with the reference strain G-186B. Referred groups are the same as in figure 1. The uninfected control (Group 8) is not shown. Four assays were performed using 12 mice for each therapeutic treatment group. Bars: Median of each group.*

Considering all the organ data obtained with the four *H. capsulatum* strains studied, significant differences in CFU values, H = 119.78 (P = 0.00001), were seen among the mouse groups under different treatments. Therefore, the untreated infected controls (group 1) were different from all infected groups that received antifungal therapy. Groups 2, 3 and 4 (treated with amphotericin B) had similar CFU values that did not resemble group 5 (treated with caspofungin). Whereas, groups 6 and 7 of infected mice (treated with the combined therapy) were different from the other tested groups, and the CFU values of these two groups were very similar (Figure 3).

Statistical analyses did not reveal significant differences in the therapy responses, H = 2.837 (P = 0.428), among the four *H. capsulatum* strains tested (data not shown).

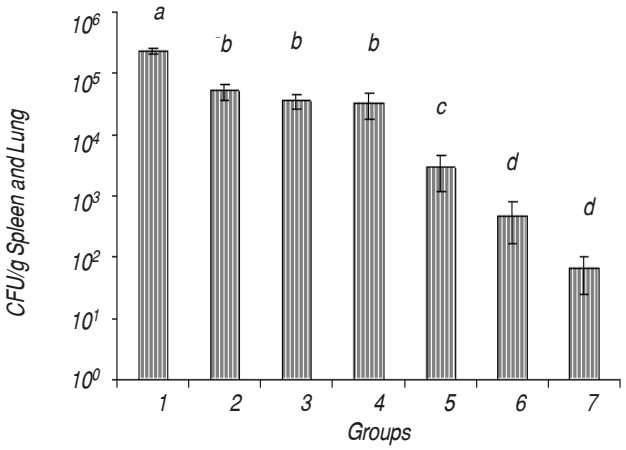

Figure 3. Average values of CFU/g in spleen and lungs after the therapeutic treatment of mice infected with the four H. capsulatum strains. The mean of CFU/g from all of the assays performed with the four strains tested are plotted. A Kruskal-Wallis variance analysis on the total values obtained with the four H. capsulatum *strains in both organs (n = 192, corresponding to n = 96 for each organ) showed significant differences in CFU values, H = 119.78 (P = 0.00001), among groups with therapeutic treatments. Using Tukey's multiple comparison test, significant differences were found among the data from therapeutic treatments. Theses differences are indicated by distinct letters, whereas the same letter indicates CFU's values with no significant differences. The mean of CFU ± SE of all assays are plotted.*

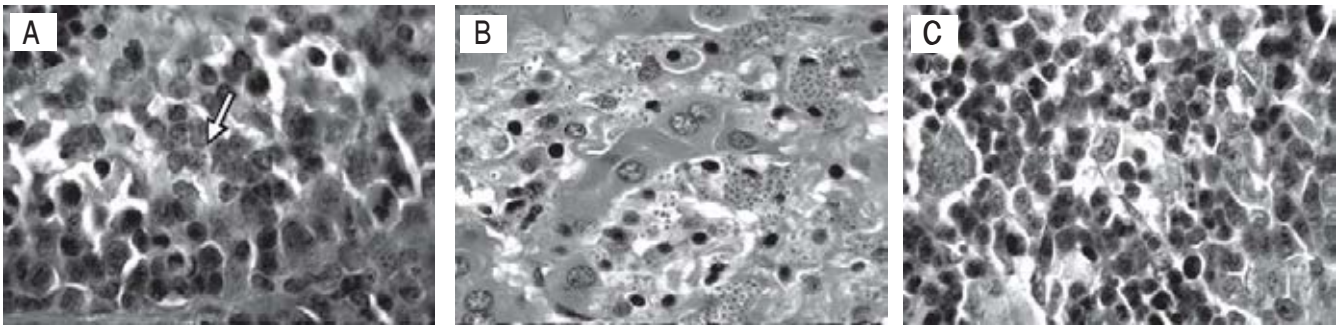

Figure 4. *Histopathology of the red pulp of selected spleen sections.* ***A.*** *Section from an infected mouse treated with combined therapy of AmB (0.5 mg/kg) and CAS (10 mg/kg), PAS stain, magnification 1000X, arrow indicates few yeast cells. B. Section from an untreated infected mouse (positive control), HE stain, and magnification 630X. C. Section from an uninfected mouse (negative control), PAS stain, and magnification 1000X.*

Histopathological observations of each organ from mice treated with monotherapy (groups 2-5, Table 1) and combined therapy (groups 6 and 7, Table 1) revealed a low fungal burden in the tissue of infected mice. As seen in figure 4a, the spleen section from an infected mouse treated with the amphotericin B and caspofungin combination shows a substantial inflammatory infiltrate with scarce yeast cells, which suggests a qualitative effect of the combined therapy. Histological section of untreated infected mouse (positive control) also showed an inflammatory response and abundant splenic macrophages with intracellular *H. capsulatum* yeasts were observed in every optical field (Figure 4b). Absence of yeast cells were confirmed in the spleen of uninfected mouse (negative control) (Figure 4c).

DISCUSSION

The disseminated clinical form of histoplasmosis is still treated with amphotericin B, despite its high toxicity. Amphotericin B is the gold standard with which new antifungal drugs are compared.[21 22] The present study demonstrates the clearance of *H. capsulatum*, from both spleen and lungs of most mice with disseminated histoplasmosis, corroborating the efficacy of the therapeutic strategy with amphotericin B and caspofungin and their combinations.

In general, mice treated with different doses of amphotericin B showed a similar clinical response to those treated with caspofungin.

The mycological evaluation revealed that all treatments resulted in a decrease in the number of CFU/g in both the spleen and lungs, when compared to untreated infected controls. However, both organs had a broad range of CFU values (0 to 3.0 x 10^5 CFU/g of tissue) in the groups treated with amphotericin B, regardless of the dose given and this occurred with the all four strains of *H. capsulatum* studied. This may be explained by natural differences in host susceptibility and the toxic effect of amphotericin B in mice, which may alter the response to the infection.

As measured by the CFU/g of each organ tested, caspofungin was a more effective therapy than amphotericin B. Although caspofungin has not been frequently used in histoplasmosis, the present results show an important fungal clearance when using caspofungin monotherapy. These findings agree with previous results described by Graybill, *et al.*,[23] although it must be emphasized that in this study a higher caspofungin dose was evaluated and the susceptibility of four *H. capsulatum* clinical strains from different geographical origins was tested. In contrast, Kohler, *et al.*[24] showed only weak efficacy of caspofungin against *H. capsulatum* in a pulmonary murine model. This was ascribed to a probable yeast phase resistance, as well as to differences in the route of infection, *H. capsulatum* strains and the mouse strain used. Additionally, while Kohler, *et al.*[24] also observed *in vitro* resistance with the yeast phase of *H. capsulatum*, Espinel-Ingroff[25] reported *in vitro H. capsulatum* susceptibility to caspofungin using its mycelial phase. This discrepancy could be associated with differences in the cell wall chemical components of each morphological phase.

Although the susceptibility of *H. capsulatum* yeast to caspofungin *in vitro* has been referred to be very low,[24] this does not necessarily imply a similar effect *in vivo*. In fact, this has been demonstrated with other antifungal agents in some fungal diseases.[25]

Considering previous data of *in vitro* assays on the resistance of *H. capsulatum* yeast to caspofungin,[24] the *in vivo* results obtained with caspofungin mono-

therapy in this study were remarkable, since the fungus was cleared from the studied organs. This finding was similar to previous studies examining caspofungin treatment in invasive pulmonary aspergillosis in rats.[26] One potential explanation for *in vitro* and *in vivo* discrepancy could be related to multiple factors, such as the host immune response and differences in the experimental animal model used.

The target for caspofungin is β1,3-D-glucan synthase, located in the plasma membrane of the fungal cell. *H. capsulatum* contains predominantly β1,3-D-glucan in its mycelial-saprobe phase and α1,3-D-glucan in its yeast-parasitic phase.[27 28] Since low amounts of β1,3-D-glucan are also found in the yeast cell wall,[28 29] it is possible that the *in vivo* inhibition of β1,3-D-glucan synthase leads to increase the fragility of the cell wall in the intraphagocytic environment. Additionally, inside the phagocyte there are mechanisms that damage the cell surface of the fungus, probably favoring the incorporation of caspofungin into the fungal plasma membrane, where the β1,3-D-glucan synthase is located. These events do not occur in the *in vitro* model, where only the yeast and the antifungal agent interact.

The administration of a combined therapy is currently considered an excellent treatment alternative in severe fungal infections, mainly in patients with predisposing factors. The effects of the combination of two drugs are widely investigated *in vitro*, in contrast to *in vivo* assays.[30-33] Based on the combined effect of antifungal agents in animal models, Johnson, *et al*.[30] and Greco, *et al*.[33] proposed the following definitions: synergistic, when the combination is better than the expected result of each antifungal; and antagonistic, when the drugs combination is worse than the expected result of individual antifungal.

Infected mice receiving the combined therapy (groups 6 and 7) showed a therapeutic efficacy, as assessed by mycological (Figures 1 and 2) and histopathological (Figure 4) findings. Based on the criteria of Johnson, *et al*.[30] and Greco, *et al*.,[33] in the present report was found a synergistic effect with the combined therapy.

The effectiveness of the combination of caspofungin and either polyenes or azoles in animal models is controversial, and fungal clearance in the host tissue has been observed in disseminated candidiasis and aspergillosis.[30 34-36] Likewise, *in vitro* assays have revealed a synergistic effect of the combined therapy with caspofungin and either amphotericin B or triazoles against *Aspergillus* and *Fusarium*.[37 38] These data support the results of the present study.

The synergistic effect of the two antifungal agents tested is probably associated with alterations of the fungal membrane permeability, due to pores formed by amphotericin B that facilitate the access of caspofungin to its target, which is also located in the plasma membrane. These two effects can destabilize the fungal cell, facilitating the fungicidal effect. The therapeutic efficiency of caspofungin and its combination with amphotericin B in experimental murine histoplasmosis is an important contribution and can set the standard for its use as salvage therapy in disseminated human histoplasmosis.

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