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INHIBITION OF XANTHINE OXIDASE BY 4-HYDROXY-6-MERCAPTOPYRAZOLO[3,4-*d*]PYRIMIDINE

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Abstract—Compound B103U, 4-hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidine, was investigated as an inhibitor of human xanthine oxidase. Studies *in vitro* demonstrated that it was significantly more potent than oxypurinol, 4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine. It formed an initial complex with electron-rich (reduced) human xanthine oxidase that was tighter than the corresponding complex formed by oxypurinol. The initial complexes with each inhibitor and reduced enzyme were internally rearranged into more stable complexes with first-order rate constants of 2.5 to 3 per min. However, the half-life of the isomerized (stable) complex with B103U was three to four times longer than the half-life of the analogous complex with oxypurinol. This stability was previously noted by Massey *et al.* (*J Biol Chem* **254**: 2837–2844, 1970) with B103U and bovine xanthine oxidase. The overall K_i values accounting for the initial and isomerized complexes were 5 nM for B103U and 100 nM for oxypurinol. B103U was also more potent as an inhibitor of bovine xanthine oxidase-catalyzed generation of superoxide radicals. Studies in mice revealed that the relative *in vitro* potency of B103U was not sustained *in vivo*. Compared to the inhibition of xanthine oxidase by oxypurinol, inhibition by B103U was neither more potent nor longer lasting. This shortcoming was not caused by weaker inhibition of mouse xanthine oxidase. Instead, it was the result of poor bioavailability. Plasma levels of available B103U rapidly decreased from samples of mouse and human blood because of reversible binding to serum proteins. B103U was also susceptible to oxidation. Two equivalents of H_2O_2 stoichiometrically oxidized the 6-thiol substituent to a sulfinic acid. This oxidized product was three orders of magnitude weaker as an inhibitor of xanthine oxidase than was B103U.

The hydroxypyrazolo[3,4-*d*]pyrimidines are interesting inhibitors of xanthine oxidase ([1–7] and reviewed in Refs 8 and 9). Allopurinol, 4-hydroxypyrazolo[3,4-*d*]pyrimidine, is used clinically to prevent the xanthine oxidase-catalyzed formation of uric acid. As an alternative-substrate inhibitor, allopurinol effectively competes with hypoxanthine and xanthine for binding to the enzyme. It is then oxidized to form oxypurinol (4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine), which is a true dead-end inhibitor of xanthine oxidase. The mechanism of inhibition by oxypurinol is unusual because its affinity is to reduced (electron-rich) rather than oxidized xanthine oxidase. Oxypurinol rapidly binds to the reduced form of the enzyme, which is generated by electrons donated from oxidizable substrates such as allopurinol, hypoxanthine or xanthine. The reduced xanthine oxidase–oxypurinol complex undergoes a slower isomerization to form a more stable inhibited complex.

Oxypurinol is useful not only as an inhibitor of uric acid formation, but also as an inhibitor of superoxide radical formation. The radical is generated as a partial by-product during the normal catalytic cycle as reduced xanthine oxidase is reoxidized by oxygen. The effectiveness of oxypurinol lies in its ability to bind reduced xanthine oxidase even in the presence of high concentrations of hypoxanthine and xanthine [10].

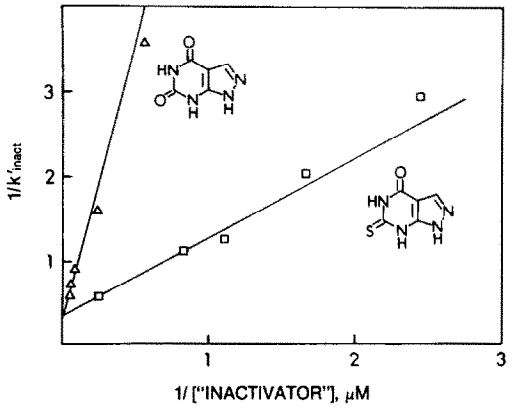

Fig. 1. Apparent inactivation of human xanthine oxidase by B103U and oxypurinol. A double-reciprocal plot of the apparent first-order rate constants (k'_{inact}) versus the concentration of inhibitor is shown. Rate constants (per min) were obtained from reaction curves for the production of uric acid from 50 μM xanthine by 0.008 units/ml of human xanthine oxidase in the presence of the indicated inhibitors: (△) oxypurinol; and (□) B103U.

The present report describes the inhibitory properties of an analog of oxypurinol, B103U (4-hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidine). This compound was originally reported to form a complex with reduced xanthine oxidase that was more stable than the analogous complex formed by oxypurinol [3]. The possibility that B103U is a more effective

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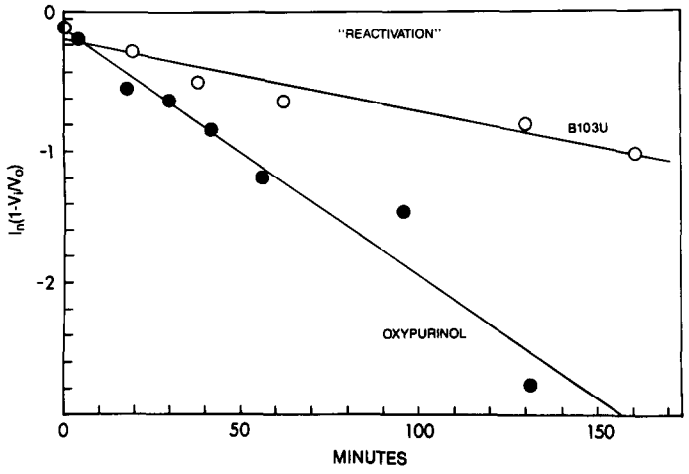

Fig. 2. "Reactivation" of human xanthine oxidase that was "inactivated" by either B103U or oxypurinol. The isolated complexes of xanthine oxidase and inhibitor were incubated at 37° and sampled at the indicated times for activity.

inhibitor of uric acid and superoxide radical production both *in vitro* and *in vivo* was investigated.

MATERIALS AND METHODS

Inhibitors. B103U and oxypurinol were synthesized at Wellcome Research Laboratories, Research Triangle Park, NC.

Xanthine oxidase assays in vitro. Human and mouse liver xanthine oxidases were purified by affinity chromatography as previously described [11]. Bovine xanthine oxidase was purchased from Boehringer Mannheim (Indianapolis, IN). Uric acid production was monitored directly at 292 nm (change in extinction = 9.75/cm/mM) with a Gilford recording spectrophotometer. The reaction temperature was electronically maintained at 37°. Reaction mixtures contained xanthine oxidase, 50 mM potassium phosphate buffer at pH 6.8, 0.3 mM EDTA, 0.1 mg bovine serum albumin/ml and 50 μM xanthine. The production of superoxide radical was monitored by following the superoxide-dependent reduction of cytochrome c at 550 nm [12]. These reaction mixtures included xanthine oxidase, 50 mM potassium phosphate buffer at pH 6.8, 0.1 mM EDTA, 120 μM hypoxanthine, 200 units catalase/ml, 80 μM cytochrome *c* [10] and inhibitors as indicated in the text. Reactions were initiated with xanthine oxidase after a 3-min preincubation. Rate constants for the development of progressive inhibition were calculated as described earlier [11, 13]. Analysis of the secondary plots of rate constant data was done according to the method of Kitz and Wilson [14] using the computer programs described by Cleland [15].

Measurement of xanthine oxidase in vivo. An assay previously developed to measure inhibition of adenosine deaminase in mice [16] was modified to measure xanthine oxidase in mice. Tritiated adenosine was replaced with [2-^{3}H]hypoxanthine (Moravek Biochemicals, Inc., Brea, CA). The release of [^{3}H]H_2O

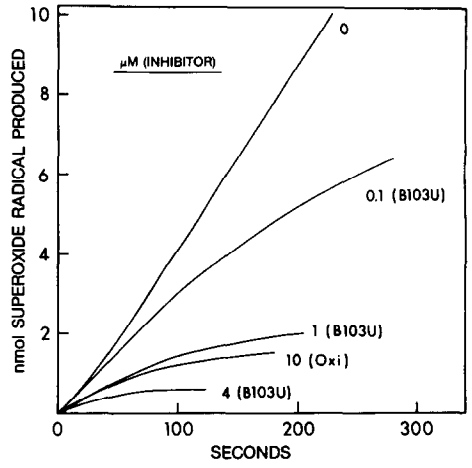

Fig. 3. Inhibition of xanthine oxidase-catalyzed production of superoxide radical by B103U or oxypurinol (Oxi). The formation of superoxide radical was monitored at 37° in the presence of 120 μM hypoxanthine, 0.1 mM EDTA, 200 units/ml catalase, 80 μM cytochrome *c*, 0.04 units/ml bovine xanthine oxidase and the indicated inhibitors.

from [2-^{3}H]hypoxanthine reflects oxidation by xanthine oxidase and aldehyde oxidase *in vivo*. Aldehyde oxidase-deficient DBA/2 mice [17] were used to minimize the contribution by aldehyde oxidase. At the given times after drug administration, 10 μCi (4–7 μmol) [2-^{3}H]hypoxanthine was injected intraperitoneally. After an additional 10 min, the animal was killed by decapitation, and blood samples were collected. The dose of [^{3}H]hypoxanthine and the pulse time were selected so that mice receiving no drug converted nearly all the tritium to [^{3}H]H_2O. The procedure for determining the amount of tritiated water in the blood is reported with the adenosine deaminase assay [16]. Since oxypurinol could

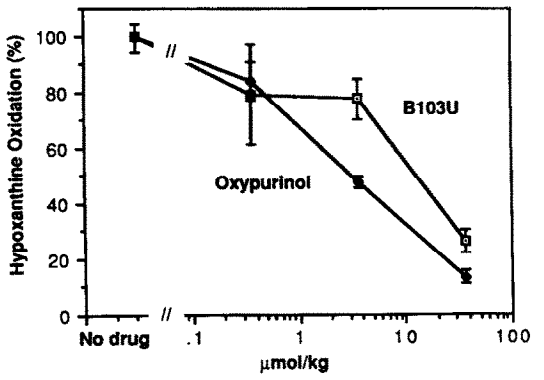

Fig. 4. Xanthine oxidase inhibition versus drug concentration. Five DBA/2 mice were injected i.p. with 0.36, 3.6 and 36 μmol/kg of B103U or oxypurinol. One hour later, [2-^{3}H]hypoxanthine was injected, and blood samples were taken after an additional 10 min. The average and standard deviation values are plotted.

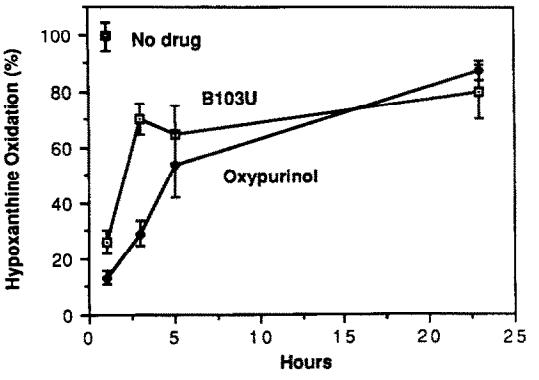

Fig. 5. Xanthine oxidase inhibition after i.p. injection of B103U or oxypurinol. Five DBA/2 mice for each time point were injected i.p. with 36 μmol/kg of B103U or oxypurinol. At 1, 3, 5 and 23 hr, [2-^{3}H]hypoxanthine was injected, and blood samples were taken after an additional 10 min. The average and standard deviation values are plotted.

inhibit the formation of $[^3H]H_2O$ by 90%, a theoretical maximum of 10% of the $[^3H]H_2O$ could arise from reactions catalyzed by enzymes other than xanthine oxidase.

HPLC. B103U and oxypurinol levels were determined by HPLC. Samples were injected onto a Microsorb C18 column (Rainin Instrument Co., Woburn, WA) and isocratically eluted with 2.4% acetonitrile in 0.1 M ammonium acetate, pH 5.5, at 1 ml/min. Concentrations of B103U and oxypurinol were calculated from their absorbance at 254 nm.

RESULTS

Inhibition of uric acid production in vitro. As previously observed with oxypurinol, B103U caused the rate of human xanthine oxidase-catalyzed oxidation of xanthine to uric acid to decelerate rapidly with time. Apparent first-order rate constants (k'_{inact}) for the decaying rates were calculated from primary data (similar to that in Fig. 6) and are presented in Fig. 1 in the double-reciprocal form. Compared to oxypurinol, significantly lower concentrations of B103U were required to cause rapid apparent inactivation. The positive y-intercepts indicate that both compounds initially form a reversible complex with the enzyme prior to an isomerization into more stable complexes [7, 14]. Dissociation constants for the initial complex (K_D) in the presence of 50 μM xanthine were calculated to be 2.8 ± 0.40 μM for B103U and 14 ± 4.1 μM for oxypurinol. The maximum k'_{inact} (k_{inact}) calculated by extrapolation to infinite concentrations of inactivator was 3.0 ± 0.24/min and 2.5 ± 0.40/min for B103U and oxypurinol respectively. These values for oxypurinol are very similar to those previously reported [7].

The rates of dissociation (reactivation) of the stable complexes of reduced xanthine oxidase with either B103U or oxypurinol were determined. Human xanthine oxidase was reduced by the addition of 20 mM dithionite under vacuum and incubated with either 30 μM B103U or 120 μM oxypurinol.

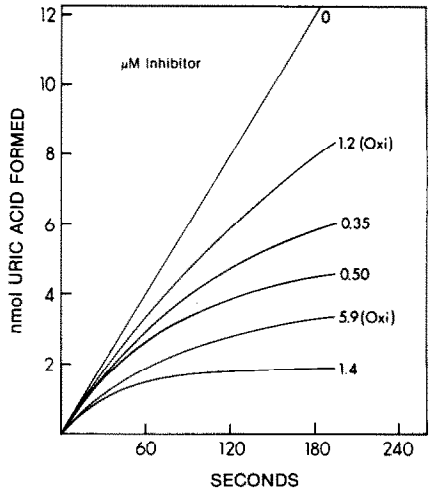

Fig. 6. Progressive inhibition of mouse xanthine oxidase. The production of uric acid from 50 μM xanthine by 0.008 units/ml of mouse xanthine oxidase was monitored in the presence of the indicated concentrations of B103U (no code) or oxypurinol (Oxi).

After 20 min at room temperature, the mixtures were chilled and the enzyme inhibitor complexes were isolated as previously described [7]. The isolated complexes were incubated at 37°, and the return of activity was assessed by assaying samples at the times indicated in Fig. 2. Rate constants for the "reactivation" of inhibited enzyme were calculated from the slopes of the lines to be 0.0054/min and 0.018/min for B103U and oxypurinol respectively.

Inhibition of superoxide radical production in vitro. The ability of B103U to inhibit the xanthine oxidase-catalyzed production of superoxide radical was studied in the presence of a high concentration of hypoxanthine. Figure 3 illustrates that submicromolar concentrations of B103U caused a rapid deceleration of the rate of radical formation and that

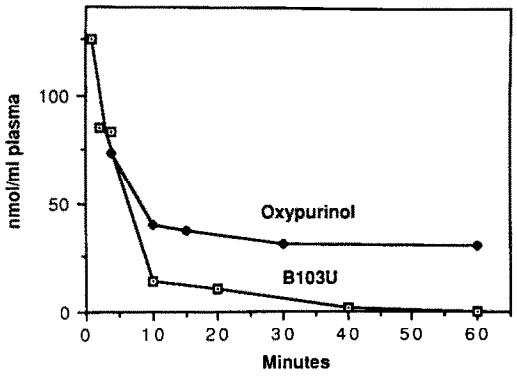

Fig. 7. Plasma levels of B103U and oxypurinol after i.v. dosing. DBA/2 mice were injected i.v. via the tail vein with 36 μmol/kg of B103U or oxypurinol. At each time point, one mouse was killed and the blood analyzed.

B103U was a considerably more potent inhibitor than oxypurinol.

Inhibition of xanthine oxidase in vivo. The potency and tenacity of B103U *in vitro* prompted a comparative study with oxypurinol in mice. Drugs were administered i.p. to circumvent potential p.o. absorption differences. Xanthine oxidase was quantitated *in vivo* by measuring the oxidation of a pulse dose of tritiated hypoxanthine. The data of Figs 4 and 5 show that *in vivo* B103U was neither more potent nor longer lasting than oxypurinol. A similar lack of superiority was noted at 1 hr after an 18 μmol/kg, i.v., dose of either compound to groups of five mice. The inhibition by oxypurinol was also at least as potent as that by B103U when assessed at the end of a multi-dose schedule of twice daily for 4 days and once on day 5 (data not shown).

Inhibition of mouse xanthine oxidase. The lack of the expected potency of B103U in mice raised the possibility that mouse xanthine oxidase may be less susceptible to inhibition than the isofunctional enzyme from human or bovine sources. To test this, the inhibition of mouse xanthine oxidase *in vitro* by B103U was studied. The data of Fig. 6 clearly show that B103U was a potent "inactivator" of this enzyme, and that the relative potency advantage of B103U over oxypurinol was similar to that observed with the human and bovine enzymes. Thus, the ineffectiveness of B103U *in vivo* was not due to less inhibitory potency with mouse xanthine oxidase.

Blood levels of B103U. To test the bioavailability of B103U, the pharmacokinetics of B103U and oxypurinol were examined in DBA/2 mice. Plasma samples were deproteinated by membrane ultrafiltration (Centrifree®, Amicon, Danvers, MA), and the levels of the respective pyrazolopyrimidines were determined by reverse phase HPLC. Mice were injected i.p. with 36 μmol/kg of either oxypurinol or B103U and then killed at 30, 60 or 180 min. Mice dosed intraperitoneally with oxypurinol had a level of oxypurinol of 28 nmol/ml plasma at 30 min following the dose, which decreased to 1 nmol/ml at 3 hr. In a comparable experiment with B103U, there was no detectable B103U (<0.1 nmol/ml) in the plasma at any time point. In addition, there was no detectable oxypurinol, a possible dethiated metabolite of B103U.

In another experiment, mice were injected with 36 μmol/kg of B103U or oxypurinol, intravenously, via the tail vein. In both experiments the levels of oxypurinol and B103U decreased rapidly between 1 and 10 min (Fig. 7). However, oxypurinol levels at 10 min were 40 nmol/ml plasma, three times higher than B103U. At 60 min after the intravenous injection, oxypurinol levels were 30 nmol/ml, whereas no measurable B103U was found.

To test if the rapid and complete disappearance of B103U from the blood was due to its binding to some filterable component of the blood, B103U was added (45 nmol/ml) to whole DBA/2 mouse blood. After a 30-min incubation at 37° and removal of high molecular weight components (>10,000 mol wt) by membrane ultrafiltration, only 40% of the drug was recovered in the ultrafiltrate.

A comparable experiment was performed to study the availability of B103U in human blood. Samples of whole blood from two donors were incubated with 45 nmol B103U/ml and analyzed after a 30-min incubation at 37°. Only 5 and 25% of the total were recovered in the two ultrafiltrates. When the ultrafiltration was performed immediately after mixing, 22% of the B103U was recovered in the ultrafiltrate. In a similar experiment when ultrafiltration was performed immediately after adding oxypurinol to human blood, 71% of the total was recovered in the ultrafiltrate.

Binding of B103U to serum proteins. Allopurinol, B103U, and oxypurinol (50–100 μM) were incubated individually with 810 μM human serum albumin (Sigma) in 0.05 M phosphate at pH 6.8 for 30 min at room temperature. The binding of each compound was quantitated by spectrophotometric estimation of the concentration of free drug recovered after ultrafiltration. By comparison to an albumin-free sample, the percentages of free allopurinol, oxypurinol and B103U were 88, 71, and 24 respectively. In a similar experiment with undiluted human serum, >90% of B103U was retained in the bound fraction. Furthermore, since all of the bound B103U was released by precipitation of the macromolecules with 3 vol. of ethanol, this binding was reversible.

Nonenzymatic oxidation of B103U by H_2O_2. Since a thiol substituent is intrinsically more reactive than an oxo substituent, it is possible that the lack of superiority of B103U *in vivo* was due to transformation to a weaker inhibitor. Therefore, the susceptibility of the thiol of B103U to oxidation by H_2O_2 was studied. Addition of H_2O_2 to a solution of B103U at pH 6.8 produced major changes in its UV absorbance spectrum. Rapid formation of an intermediate, which subsequently decayed to a stable product, was conveniently followed at 320 nm. The stoichiometry of the reaction revealed that 2 mol of peroxide were required to generate 1 mol of the final product. Oxypurinol was not oxidized by concentrations of H_2O_2 that rapidly oxidize B103U.

The product of the oxidation of B103U by H_2O_2 was isolated and characterized. NMR, mass spectral and elemental analyses demonstrated that the 6-thiol substituent was oxidized to a sulfinic acid. This oxidation product was found to be a simple competitive inhibitor of bovine xanthine oxidase with a

K_i value of 10 ± 2 μM. No time-dependent increase in inhibition was detected. In an experiment similar to the one described above for B103U, 62% of the total oxidation product was recovered in the unbound fraction from an incubation mixture with human serum albumin.

DISCUSSION

B103U is indeed a very potent inhibitor of xanthine oxidase. As judged by the positive y-axis intercept [14] of Fig. 1, it appears that its behavior is similar to that of oxypurinol in that it rapidly forms an initial complex with the enzyme. This complex then rearranges with a first-order rate constant of 3/min at 37° to form a very stable complex. Furthermore, since a substrate reductant (xanthine or hypoxanthine) or a chemical reductant (dithionite) is required to induce the tight binding, it is likely that B103U binds to a reduced form of the enzyme as does oxypurinol (see Introduction). Massey *et al.* [3] previously reported the requirement of a reductant as well as the extended half-life of the stable complex. They calculated the half-life to be 1220 min (equivalent to a first-order rate constant of 0.00057/min) at 25°. This value is in good agreement with the present value of 0.0054/min obtained at 37°, when the high temperature coefficient for this dissociation [7] is considered. Furthermore, both studies demonstrate that the half-life of the stable complex with B103U is 3- to 4-fold longer than the half-life of the complex with oxypurinol.

An overall K_i [7]* which accounts for the dissociation from the initial complex (K_D), the rate constant (k_{inact}) for the conversion of the initial complex to the stable complex, and the dissociation of the stable complex ($k_{reactivation}$) can be calculated:

$$K_i = [K_D][k_{reactivation}/(k_{inact} + k_{reactivation})]$$

The K_i values were 5 nM for B103U, and 100 nM for oxypurinol, indicating that B103U is about 20-fold more effective than oxypurinol in its overall inhibition potency. As a consequence, it is also a more potent inhibitor of xanthine oxidase-catalyzed generation of superoxide radical. Therefore, it was surprising that B103U appeared inferior to oxypurinol as an inhibitor of hypoxanthine oxidation in aldehyde oxidase-deficient mice. The data of Fig. 6 substantiate that the poor performance of B103U is not due to a diminished inhibitory potency with mouse xanthine oxidase. It is more likely a result of inferior bioavailability (Fig. 7) caused by binding of B103U to serum proteins. In particular, B103U was shown to bind to human serum albumin significantly more tightly than does oxypurinol or allopurinol. Moreover, greater than 90% of a 50 μM solution of B103U was reversibly bound to undiluted human serum. Significant binding to serum proteins has been reported previously for 4-mercaptopyrazolo[3,4-*d*]pyrimidine (thiopurinol) [18]. Neither allopurinol nor oxypurinol was found previously to bind significantly to macromolecules of human plasma [19].

It is also possible that the bioavailability of B103U is diminished by oxidative transformation. Its susceptibility to oxidation was demonstrated by the rapid two-step conversion of the 6-thiol substituent to a sulfinic acid by two equivalents of H_2O_2. This product was a simple competitive inhibitor of xanthine oxidase with a K_i of 10 μM compared to a value of 5 nM for B103U.

In conclusion, compound B103U, compared to oxypurinol, was a considerably more potent inhibitor of xanthine oxidase *in vitro* that failed to sustain its advantage *in vivo*. Decreased bioavailability due to protein binding and possibly oxidative transformation probably account for this discrepancy.

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* Although the calculations were performed correctly, this equation was misstated in the earlier report [7].

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