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Role of Cytochrome P450 Monooxygenase in the Bioactivation of Aflatoxin B1

(Peranan Sitokrom P450 Monooksigenase dalam Biopengaktifan Aflatoksin B1)

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ABSTRACT

In a previous study, the gene Eg*P450 that encodes the proteins of 505 amino acids was isolated from oil palm. The recombinant protein EgP450 is bound to phenylurea-like herbicides which detoxify the substance. Aflatoxin B1 (AFB_1), a mycotoxin produced by* Aspergillus *sp., is another toxic compound that is known to cause acute toxic effects and act as a hepatocarcinogenic agent. This study aimed to examine the role of EgP450 enzyme in mycotoxin bioactivation in human mesenchymal stem cells (hMSCs). Docking analysis showed that* Eg*P450 is bound to the group of carcinogens, which includes AFB_1, n-(2-fluorenyl) acetamide, n-n-butyl-n-butan-4-ol-nitrosamine, n-nitrosodiethylamine, n-nitrosodiethylamine and n-nitrosodimethylamine. An* in vivo *aflatoxin toxicity test on hMSCs and AFB_1 induces the expression of Bmi-1 which is one of the markers for the development of cancer. The presence of* Eg*P450 at 0.15 µg/mL could reduced the Bmi-1 expression in AFB_1 induced cells. Moreover, this protein also showed some antioxidant activity. These results exhibited the enormous potential of* Eg*P450 in the detoxification processes.*

Keywords: Carcinogen; cytochrome P450 monooxygenase; detoxification

ABSTRAK

Dalam kajian sebelum ini, gen Eg*P450 yang mengekod 505 protein asid amino telah dipencilkan daripada kelapa sawit. Protein rekombinan EgP450 terikat kepada racun herba seperti phenylurea yang menyahtoksin bahan. Aflatoksin B1 (AFB_1), mikotoksin yang dihasilkan oleh* Aspergillus *sp., adalah satu lagi toksik sebatian yang diketahui menyebabkan kesan toksik akut dan bertindak sebagai agen hepatokarsinogen. Kajian ini bertujuan untuk meneliti peranan enzim* Eg*P450 dalam biopengaktifan mikotoksin sel stem mesenkim manusia (hMSCs). Analisis dok menunjukkan bahawa EgP450 terikat kepada kumpulan karsinogen, termasuk AFB_1, n-(2-fluorenil) asetamid, n-n-butil-n-butan-4-ol-nitrosamin, n-nitrosodietilamin, n-nitrosodietilamin dan n-nitrosodimetilamin. Ujian* in vivo *ketoksikan aflatoksin ke atas hMSCs dan AFB_1 mengaruh ekspresi Bmi-1 yang merupakan salah satu penanda bagi pembangunan kanser. Kehadiran* Eg*P450 pada 0.15 µg/mL boleh mengurangkan ekspresi Bmi-1 dalam sel aruhan AFB_1. Selain itu, protein ini juga menunjukkan beberapa aktiviti antioksidan. Keputusan ini menunjukkan potensi besar* Eg*P450 dalam proses penyahtoksikan.*

Kata kunci: Karsinogen; penyahtoksikan; sitokrom P450 monooksigenase

INTRODUCTION

Aflatoxins form a major group of mycotoxin. They occur in a wide range of human foodstuffs and can cause hepatocarcinogenesis in animals (Groopman et al. 1988; Portman et al. 1970; Vesselinovitch et al. 1972; Wogan 1973). In addition, epidemiological studies showed that humans are possibly susceptible to aflatoxin induced hepatocarcinogenesis (Bulatao-Jayme et al. 1982; Groopman et al. 1988; Peers & Linsell 1973; Peers et al. 1987). Aflatoxin B1 (AFB_1) is thought to be the most carcinogenic aflatoxin. It requires the oxidation of the 8, 9 double bond of AFB_1 in order to yield the biologically active AFB_1-8,9-epoxide. AFB_1-8,9-epoxide in turn can react with DNA, whereas, microsomal cytochrome P450 (CYP)-mediated AFB_1 oxidation produces various hydroxylated metabolites such as aflatoxin Ml (AFM_1), aflatoxin Pl (AFP_1) and aflatoxin Q1, (AFQ_1). These are much less toxic than the original aflatoxins. In human liver microsomes, AFM_1 and AFQ_1 are the main oxidative detoxification products of AFB_1 (Ramsdell et al. 1991; Raney et al. 1992).

P450s are widespread in nature, i.e. they can be found in all organisms. P450 enzymes that metabolize xenobiotics play a key role in the oxidative metabolism of a wide range of exogenous as well as endogenous compounds including plant toxins, environmental carcinogens and a wide variety of anticancer drugs. P450 enzymes in human liver are, in the first place, responsible for the activation of AFB_1 to AFB_1-8,9-epoxide which is electrophilic. Several studies reported that there are five distinct human liver P450s, including 1A2, 2A6, 2B7, 3A3, 3A4 and 2A13 that can activate AFB_1 to mutagenic

metabolites and derivatives bound to DNA (Aoyama et al. 1990; He et al. 2006; Kensler et al. 2011). In the human liver, microsomes CYP3A4 is the most important P450 enzyme involved in the activation of AFB_1 to genotoxic AFB_1-8,9-epoxide or to the nontoxic 3α-hydroxy AFB_1 (Shimada et al. 1989). It has been suggested that human CYP1A2 is the P450 with high affinity that is responsible for the bioactivation of AFB_1 at low concentrations which is comparable to high concentrations in the human diet (Crespi et al. 1991). Additional data are also congruent with the hypothesis that in human liver microsomes, CYP1A2 is the main P450 involved in the oxidation of AFB_1 to AFM_1, whereas, CYP3A4 is the predominant P450 responsible for the conversion of AFB_1 to AFQ_1 (Gallagher et al. 1996, 1994; Guengerich et al. 1998).

Previously, *Eg*P450 was demonstrated as the most efficient oil palm CYP enzyme that can protect rice seedlings from exogenous herbicides of the phenylurea family. The present study aimed to characterize the role of *Eg*P450 enzyme in AFB_1 bioactivation in human stem cells which leads to the activation or detoxification of AFB_1.

MATERIALS AND METHODS

MOLECULAR DOCKING SIMULATIONS AND INTERACTION ANALYSIS

The molecular interactions docking was used for screening the *Eg*P450 with various chemicals and carcinogens using the AutoDOCK simulation tool, whereas, the *Eg*P450 is the receptor protein and set of the substrates are ligand molecules (Huey et al. 2007; Morris et al. 1996) and the SLITHER server (Lee et al. 2009).

PREPARATION AND PURIFICATION OF RECOMBINANT *EG*P450

The recombinant protein was prepared from *Escherichia coli* BL21 (DE3) harbouring *Eg*P450 according to the method previously described (Phongdara et al. 2012).

CULTURE OF HMSCS AND AFB_1 TREATMENT

hMSCs were cultured on medium containing mesenchymal stem cell growth medium (Biomed Diacnostic, Thailand) supplemented with supplement mix mesenchymal stem cell growth medium. Then 1% of penicillin/streptomycin (Biochrom, Berlin, Germany) was put into the culture which was continued in the first culture for 48 h. The culture was maintained at 5% CO_2 under the humidifier at 37°C. To determine a cytotoxic concentration of AFB_1, the stem cells were treated with chemical at concentrations of AFB_1 0 mg/mL and 0.0625 mg/mL for 24 h. In order to evaluate P450 effects (0.15, 0.30 and 0.60 μg/mL, respectively), after plating the toxin, the culture medium was mixed at the appropriate concentrations. After 24 h, culture sera was collected after the toxin treatment. Cytotoxicity was evaluated by real-time quantitative polymerase chain reaction.

BMI-1 mRNA EXPRESSION IN STEM CELLS

B-cell-specific moloney murine leukemia virus integration site 1 *(Bmi-1)*, is a prognostic marker in many cancers (Mihara et al. 2006; Song et al. 2006). *Bmi-1* expression levels were quantified in cultured cells of the hMSCs by real-time quantitative polymerase chain reaction. The mRNA levels were standardized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference housekeeping gene. The data were analyzed using $2^{-\triangle\triangle CT}$ method normalized with *GAPDH* (Livak & Schmittgen 2001).

MEASURING OF ANTIOXIDANT ACTIVITY OF *Eg*P450 BY CHEMILUMINESCENCE : XANTHINE - XANTHINE OXIDASE SYSTEM

The xanthine oxidase inhibition assay was measured spectrophotometrically by using the procedure of Vieira et al. (2009). Antioxidant activity assay was calculated using the formula:

$$\text{Antioxidant activity} = 1 - [(\text{Sample(P)} - \text{Sample(N)})/(\text{Positive control}) - (\text{Negative control})].$$

RESULTS AND DISCUSSION

MOLECULAR DOCKING SIMULATIONS AND INTERACTION ANALYSIS

The docking results clearly show that *Eg*P450 binds to carcinogens such as AFB_1, n-(2-fluorenyl) acetamide (2-AAF), n-n-butyl-n-butan-4-ol-nitrosamine (BBN) n-nitrosodiethylamine (DEN) and n-nitrosodimethylamine (DMN) (Figure 1). The lowest docking score of *Eg*P450/AFB_1 interaction model gave the best docking energy of -9.7 Kcal/mol (Table 1).

EXPRESSION AND PURIFICATION OF *Eg*P450

The analysis of *Eg*P450 enzyme of BL21 (DE3) cells on SDS-PAGE obviously showed the target size of the recombinant protein fused to the histidine-tag and a 55-kDa molecular mass (Figure 2). The bacterial cells were lysed in a lysis buffer and purified by using affinity column. Most of the soluble recombinant P450 was bound to the column and eluted in the flow through in its denatured form and subsequently allowed to refold during cut-off column.

EFFECTS OF RECOMBINANT *EG*P450 PROTEIN ON STEM CELL *IN VIVO*

In order to see the effect of AFB_1 on the stem cell hMSCs, cells were cultivated and treated with various concentrations to AFB_1. Finally, the concentration of 0.0625 was chosen in this study. Several treatments which included *Eg*P450 were designed to demonstrate the counter effect of the protein. *Bmi-1* is a prognostic marker in many cancer development. The *Bmi-1* expression interacts with several signaling containing Wnt, Akt, Notch, Hedgehog and receptor tyrosine kinase (RTK) pathway. Here, we develop a method

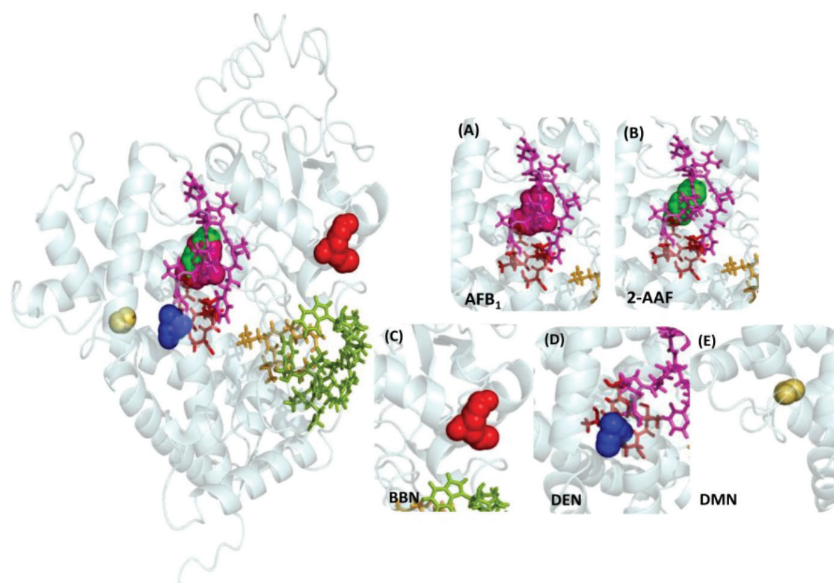
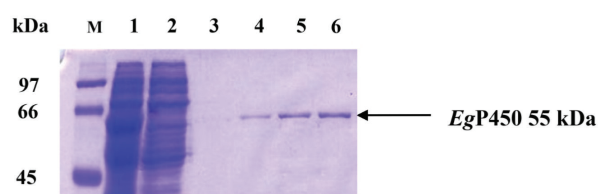


FIGURE 1. Molecular interaction models of *Eg*P450 and carcinogens. The binding of *Eg*P450 to the AFB_1 (A), n-(2-fluorenyl) acetamide (B), n-n-butyl-n-butan-4-ol-nitrosamine (C), n-nitrosodiethylamine (D) and n-nitrosodimethylamine (E)

TABLE 1. Energy table of *Eg*P450 and carcinogens interaction simulations

Chemical Name	Affinity (Kcal/Mol)
Aflatoxin B1 (AFB_1)	-8.9 – -9.7
N-(2-fluorenyl) acetamide (2-AAF)	-7.9 – -8.0
N-N-butyl-N-butan-4-ol-nitrosamine (BBN)	-4.5 – -5.1
N-nitrosodiethylamine (DEN)	-4.0 – -4.0
N-nitrosodimethylamine (DMN)	-3.3 – -3.5



*The arrow indicates the recombinant protein

FIGURE 2. Purification of *Eg*P450 as visualized on SDS-PAGE. The overexpressed protein is visible at 55 kDa after induction with IPTG. Lane 1: cell extracts, lane 2: lysis cell, lane 3: fraction after washing, lane 4-6: enzyme purification, lane M: standard of molecular weight

to screen a potential toxic and detoxification substances by measuring the expression level of *Bmi-1*.

Figure 3 demonstrates the increase of *Bmi-1* expression in hMSCs treated with AFB_1 when compared with the control (without AFB_1), whereas, the expression of *Bmi-1* is low or near normal when included at low concentration of *Eg*P450 together with AFB_1. However, at the high concentration of *Eg*P450 (0.30 and 0.60 µg/mL), *Eg*P450 helped AFB_1 to enhance the *Bmi-1* expression. These data suggest that the *Eg*P450 protein is involved in both the metabolic activation and the detoxification of AFB_1. Therefore, it is recommended to use the appropriate low concentration of *Eg*P450 in the treatment.

P450 ENZYME ANTIOXIDANT ACTIVITY

The ability of the enzyme was evaluated by Xanthine/2-methyl-6-p-methoxyphenylethynyl-imidazopyrazinone (MPEC)/xanthine oxidase (XOD) assay. The inhibition of luminescent emission caused by the decrease of superoxide anions was measured using a luminometer. Counting of photons per minute and inhibition percentage of luminescence of the samples are shown in Table 2.

The inhibition percentage of *Eg*P450 at high concentration (50 µg/mL) was superior when compared to lower concentration (20 and 35 µg/mL). However, the activity is not high when compared to the control Vitamin C.

CONCLUSION

A molecular docking analysis showed that *Eg*P450 specifically binds to carcinogens, AFB_1, n-(2-

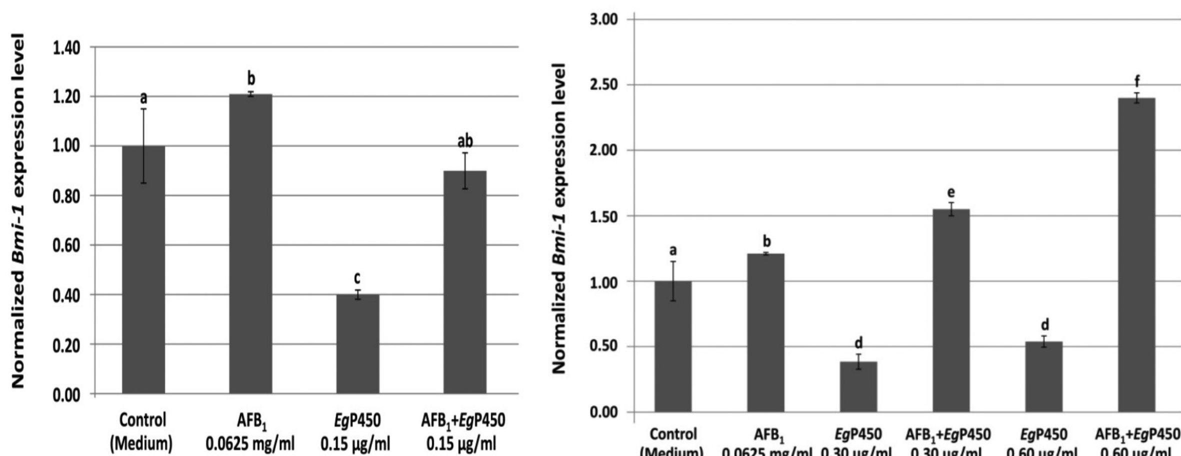


FIGURE 3. Expression of *Bmi-1* mRNA in stem cell hMSC treated with AFB_1 0.0625 mg/mL and the *Eg*P450 0.15, 0.30 and 0.60 μg/mL by real-time PCR

TABLE 2. Photon count per minute and inhibition percentage of luminescence of enzyme

Measurement	Chemiluminescence emission		% Inhibition
	Dilution buffer (N)	Enzyme solution (P)	
Negative control	7,058	77,913,538	-
Vitamin C	6,839	23,064	99.97
*Eg*P450 20 µg/mL	7,831	81,548,763	0.000
*Eg*P450 35 µg/mL	6,682	72,509,647	6.936
*Eg*P450 48 µg/mL	23,992	66,995,692	14.013

fluorenyl) acetamide (2-AAF), n-n-butyl-n-butane-4-ol-nitrosamine (BBN) n-nitrosodiethylamine (DEN) and n-nitrosodimethylamine (DMN). This is the first time a stem cell hMSCs was used to study the genotoxic of AFB_1 and its potential inhibitory effector, *Eg*P450. The study provides a screening method and showed that low concentration of *Eg*P450 possesses metabolic activity against the carciogenic effect of AFB_1.

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REFERENCES

Aoyama, T., Yamano, S., Guzelian, E.S., Gelhoin, H.V. & Gonzales, E.J. 1990. Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B1. *Proceedings of the National Academy of Sciences* 87: 4790-4793.

Bulatao-Jayme, J., Almero, E.M., Castro, M.C., Jardeleza, M.T. & Salamat, L.A. 1982. A case-control dietary study of primary liver cancer risk from aflatoxin exposure. *International Journal of Epidemiology* 11(2): 112-119.

Crespi, C.L., Penman, B.W., Steimel, D.T., Gelboin, H.V. & Gonzalez, E.J. 1991. The development of a human cell line stably expressing human CYP3A4: Role in the metabolic activation of aflatoxin B1 and comparison to CYP1A2 and CYP2A3. *Carcinogenesis* 12(2): 255-259.

Gallagher, E.P., Kunze, K.L., Stapleton, P.L. & Eaton, D.L. 1996. The kinetics of aflatoxin B1 oxidation by human cDNA-expressed and human liver microsomal cytochromes P450 1A2 and 3A4. *Toxicology and Applied Pharmacology* 141(2): 595-606.

Gallagher, E.P., Wienkers, L.C., Stapleton, P.L., Kunze, K.L. & Eaton, D.L. 1994. Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. *Cancer Research* 54(1): 101-108.

Groopman, D., Cain, L.G. & Kensler, T. 1988. Aflatoxin exposure in human populations: Measurements and relationship to cancer. *Critical Reviews in Toxicology* 19(2): 113-143.

Guengerich, F.P., Johnson, W.W., Shimada, T., Ueng, Y.F., Yamazaki, H. & Langouët, S. 1998. Activation and detoxication of aflatoxin B1. *Mutation Research* 402(1&2): 121-128.

He, X.Y., Tang, L., Wang, S.L., Cai, Q.S., Wang, J.S. & Hong, J.Y. 2006. Efficient activation of aflatoxin B1 by cytochrome P450 2A13, an enzyme predominantly expressed in human respiratory tract. *International Journal of Cancer* 118(11): 2665-2671.

Huey, R., Morris, G.M., Olson, A.J. & Goodsell, D.S. 2007. A semiempirical free energy force field with charge-based desolvation. *Journal of Computational Chemistry* 28(6): 1145-1152.

Kensler, T.W., Roebuck, B.D., Wogan, G.N. & Groopman, J.D. 2011. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicological Sciences* 120(S1): S28-S48.

Lee, P.H., Kuo, K.L., Chu, P.Y., Liu, E.M. & Lin, J.H. 2009. SLITHER: A web server for generating contiguous conformations of substrate molecules entering into deep active sites of proteins or migrating through channels in membrane transporters. *Nucleic Acids Research* 37: W559-W564.

Livak, K.J. & Schmittgen, T.D. 2001. Analysis of relative gene expression data using real- time quantitative PCR and the 2-ΔΔCT. *Methods* 25(4): 402-408.

Mihara, K., Chowdhury, M., Nakaju, N., Hidani, S., Ihara, A., Hyodo, H., Yasunaga, S., Takihara, Y. & Kimura, A. 2006. Bmi-1 is useful as a novel molecular marker for predicting progression of myelodysplastic syndrome and prognosis of the patients. *Blood* 107(1): 305-308.

Morris, G.M., Goodsell, D.S., Huey, R. & Olson, A.J. 1996. Distributed automated docking of flexible ligands - to proteins: Parallel applications of AutoDock 2.4. *Journal of Computer-Aided Molecular Design* 10(4): 293-304.

Peers, E., Bosch, X., Kaldor, J., Linsell, A. & Pluumen, M. 1987. Aflatoxin exposure, hepatitis B virus infection and liver cancer in Swaziland. *International Journal of Cancer* 39(5): 545-553.

Peers, E. & Linsell, C.A. 1973. Dietary aflatoxins and liver cancer-a population based study in Kenya. *British Journal of Cancer* 27(6): 473-483.

Phongdara, A., Nakkaew, A. & Nualkaew, S. 2012. Isolation of the detoxification enzyme EgP450 from an oil palm EST library. *Pharmaceutical Biology* 50(1): 120-127.

Portman, R.S., Plowman, K.M. & Campbell, T.C. 1970. On mechanisms affecting species susceptibility to aflatoxin. *Biochimica et Biophysica Acta* 208(3): 487-495.

Ramsdell, H.S., Parkinson, A., Eddy, A.C. & Eaton, D.L. 1991. Bioactivation of aflatoxin B1 by human liver microsomes: Role of cytochrome P450 IIIA enzymes. *Toxicology and Applied Pharmacology* 108(3): 436-447.

Raney, K.D., Shimada, T., Kim, D-H., Groopman, J.D., Harris, T.M. & Guengerich, F.P. 1992. Oxidation of aflatoxins and sterigmatocystin by human liver microsomes: Significance of aflatoxin-Q1 as a detoxication product of aflatoxin-B1. *Chemical Research in Toxicology* 5(2): 202-210.

Shimada, T. & Guengedch, E.E. 1989. Evidence for cytochrome P-450NF, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. *Proceedings of the National Academy of Sciences* 86(2): 462-465.

Song, L.B., Zeng, M.S., Liao, W.T., Zhang, L., Mo, H.Y., Liu, W.L., Shao, J.Y., Wu, Q.L., Li, M.Z., Xia, Y.F., Fu, L.W., Huang, W.L., Dimri, G.P., Band, V. & Zeng, Y.X. 2006. Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. *Cancer Research* 66(12): 6225-6232.

Vesselinovitch, S.D., Mihailovich, N., Wogan, G.N., Lombard, L.S. & Rao, K.V.N. 1972. Aflatoxin B1, a hepatocarcinogen in the infant mouse. *Cancer Research* 32: 2289-2291.

Vieira, P.M., Francisco, A.F., Souza, S.M., Malaquias, L.C., Reis, A.B., Giunchetti, R.C., Veloso, V.M., de Lana, M., Tafuri, W.L. & Carneiro, C.M. 2009. Trypanosoma cruzi: Serum levels of nitric oxide and expression of inducible nitric oxide synthase in myocardium and spleen of dogs in the acute stage of infection with metacyclic or blood trypomastigotes. *Experimental Parasitology* 121(1): 76-82.

Wogan, G.N. 1973. Host environment interactions in the etiology of cancer in man. *Cancer Research* 7: 237-241.

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