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In vitro and *in silico* effects of the polyisoprenylated benzophenones guttiferone K and oblongifolin C on P-glycoprotein function

Cherdsak Boonyong[1*], Nonthalert Lertnitikul[2], Chutichot Pattamadilok[2], Supotchana Sitthigool[2], Rutt Suttisri[2], Suree Jianmongkol[3], Angkana Wongsakul[4]

[1]Department of Medical Sciences, Faculty of Science, Pharmacology and Toxicology Unit, Rangsit University, Pathum Thani, Thailand.
[2]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.
[3]Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.
[4]School of Pharmacy, Eastern Asia University, Pathum Thani, Thailand.

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ABSTRACT

The polyisoprenylated benzophenones, guttiferone K and oblongifolin C, were found in several *Garcinia* plants (family Clusiaceae) and exhibited remarkable antitumor activity. However, their effect on P-glycoprotein (P-gp) function has never been investigated. This study aimed to assess the effect of guttiferone K and oblongifolin C on P-gp function in Caco-2 cells using the calcein-AM uptake assay to measure the accumulation of the fluorescent calcein, a substrate of P-gp, within the cells under pre/co-treatment condition. Verapamil, a well-known P-gp substrate/inhibitor, was employed as a positive control. Both compounds could inhibit P-gp function, causing increased cellular accumulation of calcein. Their roles as both P-gp substrates and P-gp inhibitors were predicted from their chemical structures through SwissADME and admetSAR programs. Furthermore, the effect of these two benzophenones on the ATP-binding region at nucleotide-binding domain 1 (NBD1) of P-gp was investigated by a molecular docking study. They could bind more favorably than verapamil to the ATP-binding region within NBD1 of P-gp. They are also bound with ATP-binding region within NBD1 of P-gp, suggesting possible inhibition of ATP hydrolysis. Therefore, guttiferone K and oblongifolin C in *Garcinia* extracts or fruits have the potential to inhibit P-gp function and might increase the risks of herb–drug interaction when used or consumed with drugs that are P-gp substrates.

INTRODUCTION

P-glycoprotein (P-gp) is one of the ATP-binding cassette (ABC) transporters and acts as an efflux pump [1]. Encoded by the ABCB1 gene, it is found on the apical surface of epithelial tissues in various organs including the brain, gastrointestinal tract, liver, and kidney, and plays roles in the absorption, distribution, metabolism, and elimination of drugs and xenobiotics from cells [2]. In cancer cells, P-gp overexpression frequently occurs, resulting in decreased intracellular concentrations of chemotherapy drugs leading to drug resistance and treatment failure [3]. Some food or medicinal substances can inhibit the function of P-gp, causing drug-drug or food/herb-drug interactions [4–6], which might benefit the therapeutic effects by increasing the accumulation of anti-cancer drugs, especially P-gp substrates, and decreasing multidrug resistance of cancer cells [7,8].

A number of reports showed the effects of chemical constituents of herbal medicines and food supplements, such as benzophenones, flavonoids, lignans, naphthoquinones, and xanthones, on P-gp activity [9–15]. For example, long-term use of *Ginkgo biloba* extracts altered the pharmacokinetics of a beta-blocker, talinolol, in healthy volunteers because flavonoids in the plant extract could inhibit P-gp function [11]. Several

*Corresponding Author
Cherdsak Boonyong, Department of Medical Sciences, Faculty of Science, Pharmacology and Toxicology Unit, Rangsit University, Pathum Thani, Thailand.
E-mail: Cherdsak.b @ rsu.ac.th

flavonoids, i.e., hesperidin, naringin, and quercetin, commonly found in fruits, vegetables, and herbal medicine, were studied *in silico* on their possible interference with P-gp function by interacting with the ATP-binding site on nucleotide-binding domain 1 (NBD1) [14]. St. John's wort (*Hypericum perforatum*), widely used for its antidepressant effect, could increase P-gp expression and its drug efflux function in peripheral blood lymphocytes of healthy volunteers [16]. On the other hand, a number of food ingredients, including piperine, capsaicin, and sesamin, could both increase the mRNA expression and inhibit the function of P-gp in vinblastine-resistant colon carcinoma (LS-180V) cells [17].

Garcinia plants (family Clusiaceae) are distributed in Asia, America, Australia, tropical and southern Africa. In Thailand, asam gelugur (*G. atroviridis*), cowa mangosteen (*G. cowa*), mangosteen (*G. mangostana*), and ma-dan (*G. schomburgkiana*) have been utilized as medicinal and edible plants [18]. They have been used in traditional medicine to treat cough, constipation, menstrual disorders, and diabetes. These plants contain flavonoids, polyisoprenylated benzophenones, and xanthones which displayed anti-inflammatory, antifungal, antioxidant, anti-human immunodeficiency virus, antilipidemic, and cytotoxic activities [19]. Guttiferone K and oblongifolin C are two polyisoprenylated benzophenones isolated from the branches, wood, and bark of *G. schomburgkiana* [18,20–22]. They were also found in other *Garcinia* species, such as the fruits of *G. yunnanensis* [7] and *G. cambogia* [23]. Although both compounds exhibited remarkable anti-cancer properties by inducing apoptosis and autophagy in *in vitro* and *in vivo* studies [18,24,25]. Their effect on the P-gp function has not been investigated. Oblongifolin C has been shown to enhance the chemosensitivity of gemcitabine-resistant pancreatic cancer to the drug [26]. In a previous study, both compounds could increase the P-gp expression in Caco-2 cells [27]. Both compounds might be able to interact with P-gp function, and the present study aimed to first demonstrate the effect of guttiferone K and oblongifolin C on P-gp function in human colorectal adenocarcinoma (Caco-2) cells and utilize the computational prediction of P-gp substrate/inhibitor and molecular docking to elucidate their mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents

Calcein acetoxymethyl ester (Calcein-AM), dimethyl sulfoxide (DMSO), Hank's balanced salt solution (HBSS), non-essential amino acids, penicillin, streptomycin, Triton X-100, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and L-glutamine from Gibco Life Technologies (Grand Island, NY). Guttiferone K and oblongifolin C (Fig. 1) were isolated from the wood of *G. schomburgkiana* as previously described [18]. Stock solutions of these two compounds and verapamil were prepared by dissolving in DMSO and stored at −20°C before use.

Cell culture

Caco-2 cell line American Type Culture Collection (ATCC® HTB-37™) was obtained from (ATCC, Rockville, MD). The cells were sub-cultured every 3 days to maintain at approximately 70% confluence and were grown in DMEM complete medium, supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin-streptomycin, and 2 mM L-glutamine, in a humidified incubator containing 5% CO_2 at 37°C [28].

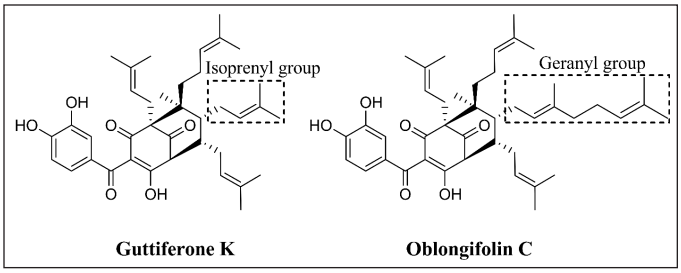

Figure 1. Chemical structures of guttiferone K and oblongifolin C.

Determination of the P-gp function

The P-gp function was determined based on the accumulation of substrate-based fluorescent probes using the calcein-AM uptake assay [15]. Caco-2 cells (passage no. 51–65) were seeded at a density of 1.3×10^4 cells/cm^2 in 24-well plates. Fresh medium was supplied to the cells for 1 day after seeding and changed every 2 days for 21 days. The cells were washed three times with HBSS and then pretreated with guttiferone K (1.25–20 µM), oblongifolin C (1.25–20 µM), or verapamil (100 µM) for 30 minutes at 37°C. Then, the P-gp substrate calcein-AM (0.4 µM) was added and the mixture was incubated for another 30-minute period as the pre/co-treatment condition. At this step, endogenous esterases were allowed to hydrolyze the non-fluorescent calcein-AM into fluorescent calcein within the cells. After this incubation period, the cells were washed with ice-cold phosphate-buffered saline to stop the reaction and lysed with 1% Triton X-100. The fluorescence intensity was measured with a microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., Hopkinton, MA) at wavelengths of 485 nm (excitation) and 535 nm (emission).

Prediction of P-gp substrate or P-gp inhibitor

The analysis of determining whether guttiferone K and oblongifolin C were P-gp substrate or P-gp inhibitor was performed computationally SwissADME and admetSAR programs [29,30]. Verapamil was used as a drug reference for P-gp substrate and inhibitor. The two-dimensional (2D) structures of compounds were drawn using ChemDraw 16 (PerkinElmer, Waltham, MA) and converted into SMILES files. These files were submitted through http://www.swissadme.ch/ and http://lmmd.ecust.edu.cn/admetsar2.

Molecular docking

Computational modeling was utilized to investigate protein-ligand interactions between NBD1 (amino acid positions 398–602) of P-gp (PDB; 4Q9H) and compounds including guttiferone K, oblongifolin C, and verapamil [14]. Water molecules were removed from P-gp structure, and hydrogen atoms were added, followed by the addition of

Gasteiger charge through AutoDoc Suite 4.2.6 (TSRI, La Jolla, CA). Three-dimensional (3D) structures of the compounds and P-gp were transformed into Protein Data Bank with partial charge Q and atom type T format files. The grid box was set at a dimension of 100 × 100 × 100 Å to cover the ATP-binding region at NBD1. Docking simulation was performed with a Lamarckian algorithm by setting default parameters at 50 times. The lowest binding energy (ΔG) and inhibition constant (Ki) were observed after docked simulation. The best ligand-protein docking was visualized as 2D and 3D intermolecular interactions using Discovery Studio 2021 Client (BIOVIA, San Diego, CA).

Statistical analysis

Data were expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed using a one-way analysis of variance, followed by Dunnett's test. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of guttiferone K and oblongifolin C on P-gp function

The effects of guttiferone K and oblongifolin C on P-gp function were evaluated in Caco-2 cells using calcein-AM uptake assay to determine the intracellular accumulation of the P-gp substrate calcein. The Caco-2 cell has been characterized as *in vitro* intestinal absorption model and used to identify the property of chemicals to be a substrate or inhibitor in guidance of the U.S. Food and Drug Administration and the European Medicines Agency [31]. Under our experimental condition, Caco-2 cells treated with the known P-gp inhibitor verapamil (100 μM) accumulated calcein at approximately three folds higher than in the control (Fig. 2). Both benzophenone-treated groups also displayed inhibition of P-gp function in a dose-dependent manner. Although at a minimum concentration of 1.25 μM both guttiferone K and oblongifolin C could not inhibit P-gp activity, significant inhibitory effect on P-gp function could be observed at higher concentrations of oblongifolin C and guttiferone K (2.5 and 10 μM, respectively). At similar concentrations of between 2.5–20 μM, oblongifolin C appeared to exhibit stronger inhibitory action on P-gp than guttiferone K. Guttiferone K, at 5, 10, and 20 μM, was about half as active as oblongifolin C. At the highest concentration of oblongifolin C (20 μM), it was able to increase the accumulation of calcein within Caco-2 cells to approximately 2 folds higher than that by verapamil.

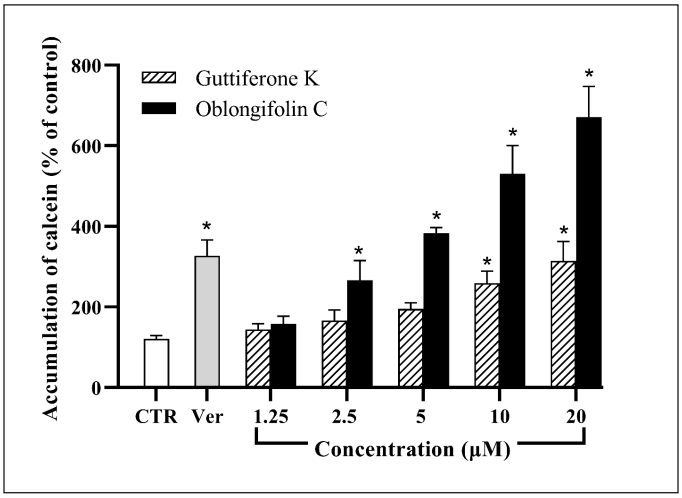

Figure 2. Effect of guttiferone K and oblongifolin C against P-gp activity in Caco-2 cells. Verapamil (Ver) at concentration of 100 μM was used as positive control. *$p < 0.05$ compared to control (CTR).

Predicting properties of guttiferone K and oblongifolin C as P-gp substrate and P-gp inhibitor

The properties of guttiferone K and oblongifolin C as P-gp substrate/inhibitor was predicted computationally by SwissADME and admetSAR programs [29,30] in order to define their role against P-gp function. Our results (Table 1) showed that both of them could be P-gp substrates and P-gp inhibitors, similar to verapamil.

Molecular docking of guttiferone K and oblongifolin C to ATP-binding region on NBD1 of P-gp

The interaction between the P-gp inhibitor and the domains of ATP-binding region on NBD1 of P-gp plays critical roles in the inference of drug efflux action [14]. In this study, the interactions of guttiferone K, oblongifolin C, and verapamil with the amino acid residues of the ATP-binding site within NBD1 were examined *in silico* [32]. As shown in Table 2, the binding free energy (ΔG) to the ATP-binding site on NBD1 of both guttiferone K (−6.87 kcal/mol) and oblongifolin C (−6.79 kcal/mol) indicated their greater affinity than verapamil (−5.46 kcal/mol). Similarly, both benzophenones displayed lower inhibitory constants (Ki) than verapamil by approximately 9–10 folds. All compounds could interact with the amino acid residues within the ATP-binding site on NBD1 via several intermolecular forces, such as hydrogen bond, alkyl-alkyl, pi-alkyl, pi-sigma, and Van der Waals interactions (Fig. 3 and Table 2). The best ligand was oblongifolin C, which could bind to the largest number of amino acids when compared to others by conventional hydrogen bonds (GLU472 and PRO473), alkyl-alkyl (VAL433, LEU439, and VAL468), pi-sigma (VAL474), and Van der Waals forces (GLN434, GLN437, SER470, GLN471, LEU475, GLU522, and LYS532). Guttiferone K also bounded to amino acids of NBD1 through alkyl-alkyl (CYS427), pi-alkyl (VAL403 and ILE405), pi-pi T-shaped (TYR397), and Van der Waals interactions (AGR400, GLN404, GLY428, GLN434, SER430, THR431, and TYP440), whereas verapamil bound to the lowest number of amino acid residues within the same binding site via alkyl-alkyl interaction (VAL468) and Van der Waals forces (GLN437, SER470, and GLU472).

Table 1. Predicted properties of guttiferone K, oblongifolin C, and verapamil as P-gp substrate and P-gp inhibitor through SwissADME and admetSAR programs.

Properties	Guttiferone K	Oblongifolin C	Verapamil
P-gp substrate	Yes	Yes	Yes
P-gp inhibitor	Yes	Yes	Yes

Table 2. Molecular interactions of guttiferone K, oblongifolin C, and verapamil with the ATP-binding region on NBD1 of P-gp.

Parameters	Guttiferone K	Oblongifolin C	Verapamil
Binding energy (ΔG; kcal/mol)	−6.87	−6.79	−5.46
Inhibitory constant (Ki; µM)	9.19	10.52	99.44
Types of interactions			
Conventional hydrogen bond	SER396, SER399, VAL403, ILE405 GLY426	GLU472, PRO473	SER905
Carbon hydrogen bond			ILE897, GLU898
Alkyl-Alkyl	TYP397, CYS427	VAL433, LEU439, VAL468, ARG901	VAL164, VAL468, LEU906
Pi-alkyl	VAL403, ILE405		
Pi-donor hydrogen			ARG901
Pi-Sigma		VAL474	
Pi-Pi T-shaped	TYR397		
Van der Waals	ASP160, AGR400, GLY428, SER430, THR431, GLN434, GLN404, TYP440, AGR901	VAL164, GLN434, GLN437, SER470, GLN471, LEU475, GLU522, LYS532, ASN899, ILE897, GLU898, PHE900, THR902, SER905	GLN437, SER470, GLU472, ASN899, PHE900, THR902,

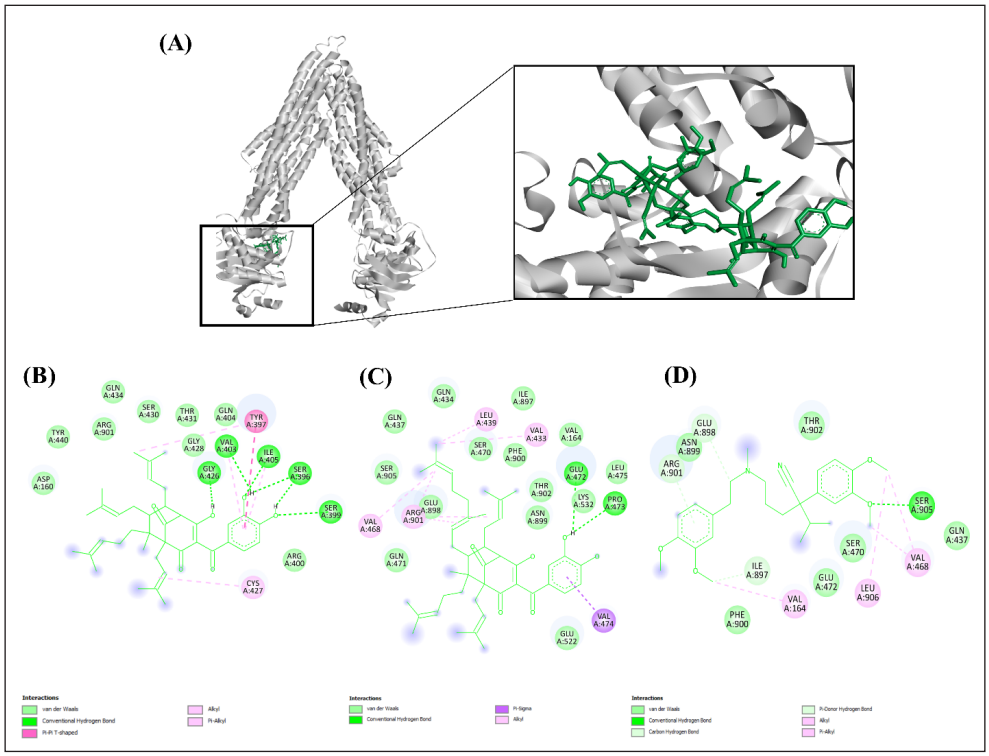

Figure 3. The 3D interaction between NBD1 of (A) P-gp and compounds. (B) The 2D interaction among guttiferone K, (C) oblongifolin C, and (D) verapamil and ATP binding site at NBD1 of P-gp.

DISCUSSION

The polyprenylated benzophenones guttiferone K and oblongifolin C are major chemical constituents of the edible fruits of several *Garcinia* species, some of which are employed as traditional medicine [7,23,33]. They are also found in the bark and twigs of *G. schomburgkiana* which are utilized in traditional Thai medicine [21]. The plant extracts rich in each compound or both of them displayed anticancer activities in various cell and animal models [18,20–22]. These benzophenones showed high potential as chemotherapeutic agents in the future [7,24,34]. Nevertheless, their effects on cellular transport proteins such as P-gp need to be further investigated.

In this study, we observed inhibition of P-gp function by both guttiferone K and oblongifolin C. At the same

concentration of 20 µM, oblongifolin C could inhibit P-gp more potently than guttiferone K by approximately three folds. Both benzophenones possess the same core structures, but with a difference in one of their isoprenylated substituents: while guttiferone K has four isoprenyl groups, one of these substituents in oblongifolin C is replaced by a geranyl group (Fig. 1). Hence, it is noticeably more hydrophobic than guttiferone K, which corresponds to their logarithm of n-octanol-water partition coefficient (log P) values of 8.2 and 7.1, respectively [24]. A similar result has been observed with geranylated chrysin (8-*C*-geranylchrysin), which showed a higher hydrophobicity index, log P value, and binding affinity than its isoprenylated analog [8-*C*-(3,3-dimethylallyl)-chrysin] [35]. Alkylation could increase the permeability of benzophenones through the cell membrane and their binding to the substrate binding site and NDBs of P-gp [36]. Therefore, more hydrophobic moiety such as a geranyl group would be an important factor to differentiate the permeability and binding affinity to P-gp of these two benzophenones.

P-gp function can be altered directly by its substrate or inhibitor. Predicting which compounds would be P-gp substrate or P-gp inhibitor is very difficult in *in vitro* and *in vivo* experiments [37,38]. The computational SwissADME and admetSAR programs are good tools to predict substrate, non-substrate, or inhibitors of P-gp based on their chemical structures. For drug discovery, these programs are also recommended for pre-screening pharmacokinetic parameters and correlating with *in vitro* and *in vivo* investigations [29,30]. In this study, both programs were utilized in order to determine the action of guttiferone K and oblongifolin C on P-gp, in comparison with verapamil, a well-known substrate and inhibitor of P-gp [39]. Our computational result indicated that, similar to verapamil, both polyprenylated benzophenones were substrates and inhibitors of P-gp, supporting previous results with benzophenone derivatives including norathyriol and benzophenone sulfonamide [36,40,41]. It is possible that these benzophenone substrates of P-gp could directly interfere with P-gp function and act as inhibitors.

Generally, P-gp function can be inhibited by several mechanisms such as blockage of the ATP binding site of NBDs in ATP hydrolysis [3,42,43]. In this study, the molecular docking experiment was used to suggest the mechanism which guttiferone K and oblogifolin C might act against NBD1 of P-gp at ATP binding. The formation of a complex structure between benzophenones and the ATP binding site of the NBD1 domain was more favorable than a verapamil-NBD1 complex, based on their observed ΔG and Ki constants. From the result of ligand-protein interaction, verapamil appeared to unfavorably bind with the ATP binding site within NBD1. Therefore, it likely interfered at the substrate binding site on the transmembrane (TMB) domains since it was a substrate of P-gp [3,43]. However, guttiferone K and oblongifolin C could readily interact with the ATP binding site within NBD1 of P-gp. These benzophenones might also interact with TMB domains because they were predicted to be P-gp substrates. Their effects on P-gp transport and ATPase activity should be further confirmed in *in vitro* study.

CONCLUSION

Two polyprenylated benzophenones, namely, guttiferone K and oblongifolin C, which are the chemical constituents of several *Garcinia* species (family Clusiaceae), were able to inhibit P-gp function in Caco-2 cells. Both compounds were predicted to be both P-gp substrates and inhibitors via SwissADME and admetSAR programs. Molecular docking analysis showed that they could bind to the ATP binding site of NBD1. Guttiferone K and oblongifolin C could thus cause herb-drug interactions when *Garcinia* plant extracts or fruits are co-administered or consumed with drugs that are P-gp substrates.

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AUTHOR CONTRIBUTIONS

Cherdsak Boonyong, Nonthalert Lernitikul, and Angkana Wongsakul: Concept and design, data acquisition, data analysis/interpretation, drafting manuscript, critical revision of manuscript, statistical analysis, supervision, and final approval. Rutt Suttisri: Material support, writing-review, and editing. Suree Jianmongkol: Material support and supervision. Chutichot Pattamadilok and Supotchanna Sitthigool: Material support. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

This original manuscript and all data are available for only research purposes from principal investigators.

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