RESEARCH ARTICLE

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[View Journal](#) | [View Issue](#) Cite this: *Med. Chem. Commun.*,
2017, 8, 415

Encapsulation of a nitric oxide donor into a liposome to boost the enhanced permeation and retention (EPR) effect†

Yu Tahara,^{‡a} Takuma Yoshikawa,^{‡b} Hikari Sato,^a Yukina Mori,^a Md Hosain Zahangir,^a Akihiro Kishimura,^{abc} Takeshi Mori^{*ab} and Yoshiki Katayama^{*abcde}

We propose a method to improve the enhanced permeability and retention (EPR) effect of nanomedicines based on tumor-specific vasodilation using a nitric oxide (NO) donor-containing liposome. NONOate, a typical NO donor, was incorporated into a PEGylated liposome to retard the protonation-induced release of NO from NONOate by the protecting lipid bilayer membrane. The NONOate-containing liposome (NONOate-LP) showed similar blood retention to an empty PEGylated liposome but almost twice the amount accumulated within the tumor. This improvement in the EPR effect is thought to have been caused by specific vasodilation in the tumor tissue by NO released from the NONOate-LP accumulated in the tumor. The improved EPR effect by NONOate-LP will be useful for the accumulation of co-administered nanomedicines.

Received 5th November 2016,
Accepted 12th December 2016

DOI: 10.1039/c6md00614k

www.rsc.org/medchemcomm

1. Introduction

Nano-sized particles are spontaneously accumulated within tumors. This phenomenon, discovered by Matsumura and Maeda and named the “enhanced permeability and retention” (EPR) effect,^{1–4} is used for passive targeting of nanomedicines. The EPR effect arises from leakage of nanoparticles from blood vessels into tumor tissue because of insufficient formation of the tight junction in the epithelium.^{5–7} Poor collection of the leaked nanoparticles by the underdeveloped lymph vessels within tumor tissues also contributes to the EPR effect.^{8–10}

Because enhancement of the EPR effect is critical to the further development of nanomedicines, several approaches to achieve this have been reported to date. Kataoka's group reported that the size of the nanomedicine entity is critical to enhancing the EPR effect, especially for intractable pancreatic cancer, because the blood vessels therein have low permeabil-

ity.¹¹ They found that polymeric micelles with a diameter of less than 100 nm show an enhanced EPR effect in the pancreatic cancer model. Kobayashi *et al.* demonstrated photo-induced necrosis of cancer cells surrounding the tumor vasculature using a near infrared-photosensitizer-modified antibody to enhance the accumulation of the nanomedicine within the tumor.^{12,13}

Enhancement of the EPR effect can be achieved by modulating the blood flow to the tumor, based on knowledge of the vascular physiology. Maeda's group reported that systemic administration of a vasoconstrictor can enhance the EPR effect.¹⁴ Normal vasculature contracts in response to vasoconstrictors to reduce blood flow, whereas tumor vasculature does not respond because it lacks the smooth muscular layer surrounding the endothelium. As a result of these differences in vascular structures, blood flow within the tumor can be selectively increased to enhance the EPR effect.¹⁵ In contrast, systemic administration of a vasodilator shows the opposite effect, *i.e.*, a decrease in blood flow to the tumor, known as the vascular steal effect.^{16–18} However, if we can selectively dilate the blood vessels feeding a tumor, the blood flow to the tumor will increase and enhance the EPR effect. Maeda's group found that the transdermal administration of nitroglycerine, which is a common vasodilator, can increase the EPR effect, although the nitroglycerine is delivered to a relatively wide region including the tumor *via* systemic circulation.¹⁹ This enhanced EPR effect is attributed to a relatively high activity within the tumor of nitrite reductase,^{20,21} which converts nitrite to nitric oxide (NO), which is a potent vasodilator. However, systemic administration of nitroglycerin was

^a Graduate School of Systems Life Sciences, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan. E-mail: ykatatcm@mail.cstm.kyushu-u.ac.jp; Tel: +81 092 802 2850

^b Department of Applied Chemistry, Faculty of Engineering, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^c International Research Center for Molecular Systems, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^d Center for Advanced Medical Innovation, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^e Department of Biomedical Engineering, Chung Yuan Christian University, 200 Chung Pei Rd., Chung Li, Taiwan, 32023 ROC

† The authors declare no competing interests.

‡ These authors contributed equally to this work.

reported to show a change of blood flow in other organs^{22,23} because of the non-specific release of NO,²⁴ which may lead to the non-specific delivery of a nanomedicine to other organs.

To induce tumor-specific vasodilation, delivery of an NO-donating vasodilator to a tumor using nanocarriers will be a promising approach. These NO-donating nanocarriers are expected to accumulate within tumor tissues *via* the EPR effect, and then release NO to dilate the blood vessels supplying the tumor, further enhancing the EPR effect. In fact, Otagiri's group developed an NO-donating recombinant protein (*S*-nitrosylated human serum albumin dimer) and found that this protein accumulated in the tumor more than a negative control protein (original human serum albumin dimer).²⁵ They recently succeeded in utilizing the recombinant protein as an enhancer of the EPR effect of co-administered nanomedicines.²⁶ The improvement of the EPR effect both in the NO-donating recombinant protein and in co-administered nanomedicines should be due to the tumor-specific vasodilation by the recombinant protein accumulated within the tumor.

In this paper, we tried to extend the concept of tumor-specific delivery of NO-donating nanocarriers to improve the EPR effect by using a typical nanocarrier, a PEGylated liposome. A common NO donor, NONOate, was selected to be incorporated into the liposome. NONOate releases NO *via* protonation-induced decomposition under physiological conditions, which is accelerated under acidic conditions.²⁷ To retard the decomposition of NONOate, we used a basic pH buffer within the interior of the liposomes (Fig. 1). The liposome membrane is known to maintain the internal pH by impeding the permeation of hydronium ions.^{28,29} In contrast, NO is a hydrophobic molecule that freely permeates the liposomal membrane.³⁰ Here, we successfully demonstrated for the first time the improvement of the EPR effect in the PEGylated liposome by incorporating an NO donor.

2. Experimental

2.1. Materials & reagents

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG) were obtained from NOF Corporation (Tokyo, Japan). Cholesterol was pur-

chased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Diethylenetriamine (DETA)-NONOate was obtained from Dojindo Laboratories (Kumamoto, Japan). 1,1'-Diocadecyltetramethyl indotricarbocyanine iodide (XenoLight DiR) was purchased from Caliper Life Sciences (Hopkinton, MA, USA). Diaminofluorescein-2 (DAF-2) was obtained from Sekisui Medical Co., Ltd (Tokyo, Japan).

2.2. Mice

Male 6 week-old ddY and male 4 week-old BALB/c mice were purchased from Kyudo Co., Ltd (Saga, Japan) and maintained under standardized conditions. Animal studies were performed with the approval of the Ethics Committee for Animal Experiments (approval no. A-26-243-0, Kyushu University, Japan) and in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan).

2.3. Preparation of NONOate-containing liposomes (NONOate-LPs)

Liposomes were prepared from solutions of the lipids (DPPC, cholesterol and DSPE-PEG) in chloroform by thin-film hydration. The molar ratio of the lipid components was DPPC:cholesterol:DSPE-PEG = 55:40:5, including 1 mol% XenoLight DiR (*vs.* total lipids) as a fluorophore. The chloroform was removed to form a lipid film with a rotary evaporator in a 50 °C water bath to maintain the temperature above the gel-liquid crystal transition temperature of DPPC (42 °C). The lipid film was then left under vacuum overnight. The dried lipid film was hydrated to produce 100 mM lipids with 100 mM *N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO) buffer (pH 9.0) by intermittently heating at 50 °C and vortexing. Liposomes were subsequently extruded through 400 nm and then 200 nm polycarbonate membranes (20 times each). DETA-NONOate (final concentration = 450 μM) was then mixed with the liposomes in 100 mM AMPSO buffer (pH 9.0) and the resulting dispersions were homogenized with an extruder at 50 °C through 100 nm polycarbonate membranes (40 times). The external medium was changed by ultracentrifugation (400 000 × *g*) for 30 min at 5 °C with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) containing 5% glucose. As negative controls, empty liposomes were prepared, dispersed in 10 mM HEPES buffer (pH 7.4) either containing or in the absence of NONOate (LP + NONOate and LP, respectively). Liposomes were stored at 5 °C and used within 24 h. The average diameter and ζ-potentials of the liposomes were determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Worcestershire, UK). The amount of incorporated NONOate in NONOate-LP was quantified by using DAF-2 to detect NO released from the liposomes for 7000 min in PBS (pH 7.4) at 37 °C. The NO release was found to be saturated at 7000 min.

Fig. 1 Retardation of NO release from NONOate-containing liposomes by modulation of the internal pH of the liposomes.

2.4. NO release profile from NONOate-LP

NONOate-LP was prepared by the method described above (to a final concentration of DETA-NONOate of 200 μM). A solution of NONOate-LP (lipid concentration = 40 mM) was mixed with an equal volume of 50 μM DAF-2 solution under physiological conditions (pH 7.4, 37 $^{\circ}\text{C}$). At each time point, the fluorescence intensity of DAF-2T, which is the product of the reaction between DAF-2 and NO released from NONOate-LP (excitation = 495 nm; emission = 515 nm), was measured using a FP-8600 spectrofluorometer (JASCO International Co., Ltd., Tokyo, Japan). The percentage of NO released was calculated by $[(F_{I_t} - F_{I_0}) / (F_{I_{7000\text{min}}} - F_{I_0})] \times 100\%$, where F_{I_0} , F_{I_t} , and $F_{I_{7000\text{min}}}$ represent the fluorescence intensity of DAF-2T at incubation times of 0, t , and 7000 min (when NO was completely released from NONOate-LP), respectively.

2.5. Cell culture

A murine colon adenocarcinoma cell line, CT-26, was cultured in RPMI 1640 medium. This medium contained 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, and 0.25 $\mu\text{g mL}^{-1}$ amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY, USA). The cells were harvested in a humidified atmosphere containing 5% CO_2 in air at 37 $^{\circ}\text{C}$.

2.6. Measurement of systemic blood pressure

The mice (male 7 week-old BALB/c, $n = 3$) were intravenously (i.v.) injected through the tail vein with liposomes encapsulating 0.1, 1, 10, or 100 nmol of DETA-NONOate at 2 μmol of lipid in a 100 μL volume of 10 mM HEPES buffer containing 5% glucose. 20 h after injection, the tails of the mice were fitted with a tail-cuff microsensor device (model MK-2000ST; Muromachi Kikai, Tokyo, Japan) and the systemic blood pressure was measured.

2.7. Blood circulation

To compare the blood circulation half-life of NONOate-LP with the empty LP, mice (male 7 week-old ddY, $n = 24$) were randomly divided into eight groups. Each group of mice was i.v. injected with NONOate-LP encapsulating 1 nmol of DETA-NONOate or empty LP. At 2, 8, 24 and 48 h following administration, blood was collected from three mice in each group. The blood was allowed to clot for 30 min at room temperature and then for 24 h at 4 $^{\circ}\text{C}$, and the serum was separated by centrifugation at 3000 rpm for 20 min at 4 $^{\circ}\text{C}$. The amount of liposomes in the serum was determined by the fluorescence intensity of each sample, using the fluorescence intensity resulting from DiR. Serial dilution of liposomes was carried out to obtain a standard calibration curve. A blank serum sample without liposome injection was measured to determine the serum autofluorescence level, which was subtracted from the fluorescence intensities of the injected samples during the

concentration calculation. The liposomes are presented as the percentage of injected dose per mL of serum.

2.8. Nephrotoxicity and hepatotoxicity

To evaluate the nephrotoxicity and hepatotoxicity of NONOate-LP, mice (male 7 week-old ddY, $n = 12$) were randomly divided into four groups. At 24 h following administration of NONOate-LP, empty LP, or LP mixed with 10 nmol of DETA-NONOate, blood was collected from three mice in each group. The blood was allowed to clot for 30 min at room temperature and for 24 h at 4 $^{\circ}\text{C}$, and the serum was separated by centrifugation at 3000 rpm for 20 min at 4 $^{\circ}\text{C}$ for investigation. Serum creatinine and aspartate aminotransferase (AST) concentrations were determined using the LabAssay Creatinine (Wako Pure Chemical Industries, Osaka, Japan) and Transaminase C2- Test Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively.

2.9. The evaluation of LP accumulation within tumors

To evaluate the enhancement of LP accumulation in tumors by NO, mice (male 6 week-old BALB/c, $n = 21$) were randomly divided into three groups. The mice were inoculated with a subcutaneous injection of 1×10^6 CT-26 cells suspended in 100 μL of Hank's balanced salt solution (HBSS, Gibco Invitrogen Co.) into the dorsum after removal of their hair from the lower half of the dorsum with an electric hair clipper. Tumors were allowed to grow to approximately 100 mm^3 . Tumor volume (V) was measured and calculated using the following formula: $V (\text{mm}^3) = (I \times W^2)/2$, where I and W indicate the long and short dimensions of the tumor tissue, respectively. Each group of mice was i.v. injected with 100 μL of NONOate-LP containing 1 nmol of DETA-NONOate, empty LP, or LP mixed with 10 nmol of NONOate. At 24 and 48 h following administration, the mice were imaged for DiR dye fluorescence using an IVIS® Lumina instrument (Xenogen, Alameda, CA, USA). The tumors and organs, including the heart, liver, spleen, lung and kidney, were harvested and rinsed with Dulbecco's phosphate-buffered saline (DPBS). Each organ was imaged for DiR dye fluorescence using the IVIS® Lumina instrument. The harvested tumors were lysed using lysis buffer (100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.05% Triton-X100, pH 7.8) for quantitative analysis of accumulated LPs.

2.10. Statistical analysis

The Student's t -test was applied in the analysis of the effect of the amount of NONOate-LP on blood pressure in mice, fluorescence intensity of the tumor region and fluorescence intensity of the tumor lysate.

3. Results and discussion

3.1. Preparation of liposomes

NONOates are complexes between NO and alkylamines, and spontaneously release NO *via* protonation-induced decomposition, a process that is accelerated under acidic conditions.²⁷ The half-life of NONOates varies with the structure of alkylamines.³¹ Here, we chose diethylenetriamine (DETA)-NONOate, which has the longest half-life amongst the reported NONOates (>10 h at neutral pH).^{31,32} NONOate was incorporated into PEGylated liposome during the extrusion process, following the previously described procedures.²⁸ To retard the protonation-induced decomposition of incorporated NONOate, AMPSO buffer with a basic pH (100 mM AMPSO, pH 9.0) was used as a medium in the interior of the liposomes because NONOate is stable at basic pH. The obtained liposomes were suspended in neutral buffer at 5 °C, which is well below the gel-liquid crystalline phase transition temperature of the matrix lipid, DPPC (42 °C).^{28,33} We also prepared two types of negative control, empty LP and LP mixed with 10 nM DETA-NONOate (LP + NONOate). The characteristics of the liposomes are summarized in Table 1. The diameter, polydispersity index (PDI), and ζ -potential of these three types of liposomes were closely similar. Their diameters, *ca.* 130 nm, were suitable for tumor accumulation by the EPR effect.³⁴

The NO release profile from NONOate-LP was measured using a fluorescent probe for NO, DAF-2.³⁵ After NONOate is decomposed, the released NO, which is a hydrophobic gas, freely permeates the liposomal membrane to react with DAF-2 to form a fluorescent adduct.

As shown in Fig. 2, NO release from NONOate-LP was slightly delayed compared with that from free NONOate. The half-life for NONOate hydrolysis was extended from 21 to 35 h by incorporating it into liposomes because the basic pH was maintained inside the liposomes by the lipid bilayer membrane.

3.2. Blood half-life of NONOate-LP and effect of dose on blood pressure

Blood half-lives of LP and NONOate-LP were examined after i.v. injection into mice. As shown in Fig. 3, the blood clearance profiles of both LP and NONOate-LP were almost superimposable. The blood half-life was estimated to be about 12 h for both liposomes, which is consistent with the reported half-life of PEGylated liposomes in mice.^{33,36} Thus, the incorporation of NONOate into liposomes did not affect the blood half-life of PEGylated liposomes. It is notable that the half-

Fig. 2 NO release profile of free NONOate and NONOate-LP in PBS (pH 7.4), measured using DAF-2 at 37 °C. Results are expressed as mean $\pm$ SE ($n = 3$).

life for NONOate decomposition of NONOate-LP (35 h) is comparable to the blood half-life of NONOate-LP. Thus, the injected NONOate-LP continuously releases NO into both the blood and accumulated tissues until NONOate-LP has almost completely disappeared from the blood.

We then examined the effect of NONOate-LP dose on systemic blood pressure to determine the optimal dose, which would not change the systemic blood pressure. The blood pressure was measured 20 h after injection, at which time about 20% of liposomes were expected to be retained in the blood circulation according to Fig. 3. As shown in Fig. 4, a 100 nmol dose of NONOate resulted in a decrease in blood pressure, while below that amount, the blood pressure remained constant, irrespective of the amount injected. We subsequently used a 1 nmol NONOate dose, which was well below the critical dose, to avoid changes in systemic blood pressure.

3.3. Tumor accumulation of NONOate-LP

We then checked the tumor accumulation of NONOate-LP in tumor-xenografted mice. Fluorescence images of seven mice

Fig. 3 Concentration change of liposomes in mouse serum after intravenous injection into mice. Values at each time point are the average for three mice. The dose of NONOate-liposome was 1 nmol of NONOate. Data are expressed as mean $\pm$ SD ($n = 3$).

Table 1 Characteristics of liposomes

Sample	Diameter (nm)	PDI	ζ -Potential (mV)
LP	130	0.101	-19
LP + NONOate	132	0.107	-18
NONOate-LP	128	0.111	-18

Fig. 4 Effect of the amount of NONOate-LP on blood pressure in mice. NONOate-LP was intravenously injected into mice and the blood pressure was measured after 20 h. Results are expressed as mean $\pm$ SD ($n = 3$). Statistical results were compared with those obtained without treatment. ***: $P < 0.001$. N.S.: not significant.

from each group 48 h after i.v. injection are summarized in Fig. 5A. An enhanced accumulation of liposomes was ob-

served in the case of NONOate-LP (containing 1 nmol of NONOate) compared with LP and LP + NONOate. Fig. 5B summarizes the average fluorescence in the tumor region for seven mice from each group. The fluorescence intensity of LP + NONOate was similar to that of LP, indicating that a 10 nmol dose of free NONOate did not affect the tumor accumulation of liposomes. However, NONOate-LP showed an increase in accumulation from LP to 60% and 70% at 24 and 48 h after injection, respectively.

Fig. 5C shows the fluorescence images of each organ resected from mice 48 h after liposome injection. Compared with the control groups (LP and LP + NONOate), NONOate-LP showed intense accumulation in the tumor while the accumulation in other organs remained at a constant level. The fluorescence intensities of the tumor lysate from each group are summarized in Fig. 5D. An 80% increase in fluorescence was observed for NONOate-LP compared with LP. The amounts of accumulated liposomes per total dose were calculated to be 5% for the controls (LP and LP + NONOate) and 9% for NONOate-LP. Thus, it is clear that NO released from

Fig. 5 Biodistribution of liposomes in BALB/c mice xenografted with CT-26 tumors. (A) IVIS images of fluorescence resulting from liposomes 48 h after intravenous injection. (B) Fluorescence intensity of the tumor region of an average of seven mice from each group 24 (open bars) and 48 h (black bars) after injection. Results are expressed as mean $\pm$ SD ($n = 7$). (C) Fluorescence images of each organ resected from mice 48 h after liposome injection (B: blood, H: heart, K: kidney, Li: liver, Lu: lung, S: spleen, T: tumor). (D) Fluorescence intensity of tumor lysate for each liposome 48 h after injection. Results are expressed as mean $\pm$ SD ($n = 3$). ***: $P < 0.001$.

NONOate-LP improves tumor accumulation of liposomes *via* the EPR effect.

3.4. Toxicity of NONOate-LP

We examined the toxicity of NONOate-LP towards the kidney and liver by creatinine clearance and AST activity, respectively. As shown in Fig. 6, the toxicity of NONOate-LP was negligibly low, similar to those of LP and LP + NONOate despite the level of accumulation in the kidney and liver, as observed in Fig. 5C. Thus, the amount of NO released from NONOate-LP was not at a toxic level for these organs. The non-toxicity of NONOate-LP in the present dose (1 nmol) is supported by a previous report that examined the toxic concentration of DETA-NONOate.³⁷

3.5. Mechanism of improved EPR effect in NONOate-LP

The improved tumor accumulation of NONOate-LP observed in Fig. 5 could be ascribed to NO released from NONOate-LP. The mechanism of the improved tumor accumulation by NO is explained below, as shown in Fig. 7, based on the results obtained here. Once i.v. injected, NONOate-LP starts to accumulate in the tumor *via* the EPR effect. The time required for tumor accumulation of PEGylated liposomes has not been reported to date, but some nanoparticles were reported to begin tumor accumulation rapidly after i.v. injection (<1 min).^{38,39} The NONOate-LP accumulated in the tumor will continuously release NO to raise the NO concentration in the tumor region for an extended period of time (the half-life for NO release is 35 h, according to Fig. 2). Thereby, vasodilation in the tumor region takes place, increasing blood flow to the tumor, which leads to the enhancement of the EPR effect for further NONOate-LP that remains in the blood. The accumulated NONOate-LP further raises the concentration of NO in the tumor region to maintain or to enhance the vasodilation in the tumor region. Due to the short lifetime of NO in a biological fluid (half-life: ≤ 2 s (ref. 40)), NO concentration will be kept low not to induce cytotoxicity. This sequence of tumor-specific vasodilation and enhancement of the EPR effect can thus form a positive feedback mechanism to amplify the EPR effect.

Fig. 6 Toxicity of liposomes towards the kidney and liver, estimated by creatinine clearance (A) and AST activity (B), respectively. Toxicity was measured 24 h after intravenous injection of each liposome. Results are expressed as mean $\pm$ SD ($n = 3$). N.S.: not significant.

Fig. 7 Expected mechanism behind the improved EPR effect observed in tumor accumulation of NONOate-LP.

4. Conclusions

Here, we successfully extended the concept of tumor-specific delivery of NO-donating nanocarriers to improve the EPR effect by using a representative nanocarrier of a PEGylated liposome. We prepared NONOate-containing PEGylated liposomes (NONOate-LP) that showed higher sustained release of NO than free NONOate because of the retardation of hydrolysis by the liposomal membrane. The NONOate-LP showed almost double the accumulation in tumors compared with empty PEGylated liposomes (LP), while maintaining a constant level of accumulation in other organs. This improvement in tumor-specific biodistribution of NONOate-LP was achieved using a relatively small dose of NONOate, which elicited neither a change in systemic blood pressure nor detectable toxicity. NONOate-LP will be useful for increasing the accumulation of co-administered nanomedicines.

Acknowledgements

We thank Prof. Hiroshi Maeda (Sojo University) for fruitful discussions. We appreciate assistance from Prof. Toyoshi Iguchi (Kyushu University) with blood pressure measurements. We are also grateful for financial support from a Grant-in-Aid for Challenging Exploratory Research (25560202) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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