Asia Oceania Journal of Nuclear Medicine & Biology
http://aojnmb.mums.ac.ir
AOJNMB

Evaluation of D-isomer of ^{18}F-FBPA for oncology PET focusing on the differentiation of glioma and inflammation

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ARTICLEINFO

Article type:
Original Article

Article history:
Received: 3 Apr 2020
Revised: 28 May 2020
Accepted: 5 Jun 2020

Keywords:
FBPA
Small animal
LAT1
C6 glioma
Inflammation

ABSTRACT

Objective(s): L-4-borono-2-^{18}F-fluoro-phenylalanine (L-[^{18}F]FBPA), a substrate of L-type amino acid transporter 1 (LAT1), is a tumor-specific probe used in positron emission tomography (PET). On the other hand, it has not been examined whether another isomer D-[^{18}F]FBPA accumulates specifically in the tumor. Here, we compared the accumulation of D-[^{18}F]FBPA in C6 glioma and inflammation to evaluate the performance of D-[^{18}F]FBPA as a tumor-specific probe.

Methods: HEK293-LAT1 and HEK293-LAT2 cells were tested for [^{14}C]-leucine or [^{14}C]-alanine transport, and IC_{50} values of L- and D-FBPA were evaluated in both cell types. PET was conducted in rat xenograft model of C6 glioma with LAT1 expression and model of turpentine oil-induced subcutaneous inflammation (n=10 for both models). The concentrations of D-[^{18}F]FBPA were compared between glioma and inflammatory lesion using standardized uptake value (SUV) .

Results: In contrast to L-FBPA, which inhibited substrate uptake in both HEK293-LAT1 and -LAT2 cells, D-FBPA showed no inhibitory effect on both cells, suggesting low transporter selectivity of D-[^{18}F]FBPA against LAT1 and LAT2. Static PET analysis showed low accumulation of D-[^{18}F]FBPA in C6 glioma and inflammatory lesion (SUV_{max}=0.80±0.16, 0.56±0.09, respectively). Although there was a statistical difference in SUV_{max} between these tissues, it was difficult to distinguish glioma from inflammation on the PET image due to its low uptake level. Therefore, it was suggested that D-[^{18}F]FBPA is not a suitable tumor-specific probe for oncology PET in contrast to L-[^{18}F]FBPA.

Conclusion: This study demonstrated that D-[^{18}F]FBPA is not a LAT1-specific PET probe and shows low uptake in C6 glioma, indicating its unsuitability as a tumor diagnosis PET probe.

Please cite this paper as:

Hirai N, Watabe Tadashi, Nagamori Sh, Wiriyasermkul P, Tanaka Y, Victor Romanov1, Naka S, Kanai Y, Liu Y, Tani N, Sakai T, Tatsumi M, Shimosegawa E, Kanai Y, Hatazawa J. Evaluation of D-isomer of ^{18}F-FBPA for oncology PET focusing on the differentiation of glioma and inflammation. Asia Ocean J Nucl Med Biol. 2020; 8(2):102-108. doi: 10.22038/AOJNMB.2020.47399.1321

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Introduction

The positron emission tomography (PET) probe, 4-borono-2-[^{18}F]-L-phenylalanine (L-[^{18}F]FBPA) has been used for the pretreatment evaluation of boron neutron capture therapy (BNCT) (1). In BNCT, L-borono-phenylalanine (BPA) is infused to the patients with refractory cancers, and neutron beams are irradiated into the tumor to induce the treatment effect by alpha rays and recoil nucleus of lithium emitted by the nuclear reaction of ^{10}B and neutron (2). Aside from its original purpose, L-[^{18}F]FBPA has recently garnered attention as a tumor diagnosis PET probe because of its high selectivity to L-type amino acid transporter 1 (LAT1) (3). LAT1 expression is observed in various types of cancers and is related to the degree of malignancy and poor patient survival (4–7). Moreover, LAT1 shows minimal expression in normal tissues and low accumulation in inflammatory lesions (4, 8, 9). Our group demonstrated in the previous preclinical study that L-[^{18}F]FBPA is very useful in distinguishing tumor from inflammation (3).

However, it is not yet reported whether PET imaging with the D-isomer of [^{18}F] FBPA (D-[^{18}F]FBPA) can distinguish tumor from non-tumor tissue. Some D-isomers of amino acid tracers, including D-[^{18}F]FBPA, are recently reported to show high tumor to normal brain ratio (TBR) or tumor to blood ratio due to low accumulation in background tissues (10–12). As indicated in these previous studies, D-[^{18}F]FBPA may be a better tumor imaging PET probe than L-[^{18}F]FBPA, but it is still necessary to verify that D-[^{18}F]FBPA shows specific accumulation in tumor, and not in non-tumor tissue .

Therefore, our purpose was to evaluate the potential of D-[^{18}F]FBPA as a tumor diagnosis probe. In this study, we evaluated the selectivity of D-FBPA to LAT1 by conducting in vitro cellular uptake experiments. We also compared the accumulation of D-[^{18}F]FBPA in C6 glioma and inflammatory lesion *in vivo*.

Methods

In vitro cellular uptake experiments

HEK293 stably expressing hLAT1 (HEK293-LAT1) and hLAT2 (HEK293-LAT2) were constructed and maintained as previously described (13). The cells were seeded at 2 × 10^5 cells/well in poly-D-lysine coated 24-well plate and cultured for 2 days. Uptake reaction was initiated by incubation of the cells for 1 min in Na^+-free-Hank's balanced salt solution (HBSS) containing 1 μM L-[^{14}C]leucine (0.06 Ci/mmol; Moravek Biochemicals, Brea, USA) or L-[^{14}C]alanine (0.53 Ci/mmol; American Radiolabeled Chemicals, St. Louis, USA) for HEK293-LAT1 and HEK293-LAT2, respectively. Inhibitory effect of L- or D-FBPA on the uptake was evaluated by adding the test compounds at the same time with the radioisotope-labeled substrates. After incubation, the reaction was stopped by ice-cold Na^+-free-HBSS. Radioactivity inside the cells was monitored using a β-scintillation counter. The uptake data were fitted to non-linear regression in GraphPad Prism 8.0 (GraphPad Software, La Jolla, USA) .

Synthesis of D-[^{18}F]FBPA

The [^{18}F]FBPA was synthesized as previously reported (1). Briefly, ^{18}F-acetylhypofluorite was reacted with 30 mg of 4-borono-D-phenylalanine (for D-[^{18}F]FBPA) in 5 mL trifluoroacetic acid (TFA). After that, TFA was removed with vacuum using 200 mL/min of N_2 gas flow, and the residue was dissolved in 0.1% acetic acid. The solution was then injected into semi-preparative high-performance liquid chromatography (HPLC) and the [^{18}F]FBPA fraction (retention time; 18-20 min) was collected. The fraction was dried using an evaporator and then dissolved in normal saline. The radiochemical purity and the optical purity of D-[^{18}F]FBPA was > 99% and >98%, respectively, as analyzed by HPLC.

Animal preparation

Male F344 rats and male Wistar rats were purchased from Charles River Japan, Inc. (Atsugi, Japan) and SLC (Hamamatsu, Japan), respectively. They were housed under a 12-h light/12-h dark cycle, with food and water available ad libitum. C6 glioma cell line, which was derived from rat glioma induced by N-nitrosomethylurea, was procured from RIKEN BRC (Tsukuba, Japan). The C6 glioma cells were cultivated on minimal essential medium supplemented with 10% fetal bovine serum, in an incubator filled with 5% CO_2 at 37 °C. The cultured cells were released from the culture surface with 0.25% trypsin, and 0.2 ml mixture of the C6 glioma (1.2-1.3×10^6 cells) and Matrigel was subcutaneously injected into the bilateral shoulders of F344 rats (n=10) to establish tumor xenograft models (3). Besides, 0.2 ml of turpentine oil was subcutaneously injected at the bilateral shoulders of Wistar rats (n=10) to establish inflammation models according to the previous study (3).

PET and data analysis

Rat xenograft models of C6 glioma (n=10, body weight 201.7±11.4 g) and models of turpentine oil-induced subcutaneous inflammation (n=10, body weight 190.6±9.0 g) were investigated in this study. PET experiments were conducted 2 weeks

after the xenotransplantation in the tumor xenograft model, and 4 days after the turpentine oil injection in the inflammation model. Under isoflurane anesthesia, D-[^{18}F]FBPA solution was injected into the normal rats through the tail vein (C6 glioma models: 37.7±5.1 MBq, inflammation models: 35.4±6.1 MBq). Micro-PET (Inveon PET/CT, Siemens, Munich, Germany) was used for PET scanning of the small animal (14). The 70-min dynamic PET scans were started at the same time as the D-[^{18}F]FBPA injection (n=3 for each model), and 10 min static PET scans were conducted 60 min after the injection (n=7 for each model). The concentration of D-[^{18}F]FBPA was measured using standardized uptake value (SUV), which is the corrected unit of radioactivity based on the injection dose (MBq) and body weight (g). Ellipsoid volumes of interest (VOIs) were placed on the liver, brain, heart, lungs, muscle, kidneys, and pancreas to investigate the whole-body distribution of D-[^{18}F]FBPA, using PMOD software (ver 3.601; PMOD Technologies LLC, Zurich, Switzerland). We also set VOIs on C6 glioma and inflammatory lesions. We calculated target to muscle ratio (T/M ratio), which is defined by the ratio of SUV_{max} of the target, such as C6 glioma or inflammatory lesion, to SUV_{mean} of the muscle. The time-activity curve (TAC), maximum or mean value of SUV (SUV_{max} or SUV_{mean}), and T/M ratio were compared between these two tissues .

Histological analysis

After PET imaging, the animals were sacrificed by euthanasia under deep isoflurane anesthesia. The glioma and inflammatory lesion were excised and fixed with 4% paraformaldehyde, after which they were frozen to be sliced with a cryostat microtome. The tissue slices of the glioma and inflammatory lesion were stained with hematoxylin and eosin (H&E) and examined using light microscopy (Keyence, Osaka, Japan).

Statistical analysis

Comparisons between values of two groups were performed by unpaired t-test using Excel. A *P* value <0.05% was considered to indicate a significant difference.

Results

We conducted *in vitro* cellular inhibition experiments and compared the IC_{50} between L- and D-FBPA. D-FBPA did not show inhibitory effect in HEK293-LAT1 or HEK293-LAT2 cells (Figure 1a, c). The inhibition curve of D-FBPA could not be fitted to non-linear regression. In contrast, L-FBPA inhibited L-[^{14}C] leucine or L-[^{14}C] alanine in both HEK293-LAT1 and HEK293-LAT2 cells, respectively. The IC_{50} of L-FBPA effect on LAT1 and LAT2 were 200.3 and 498.4 μM, respectively (Figure 1b, d). Thus, we hypothesized that D-FBPA is not a substrate of LAT1 and LAT2.

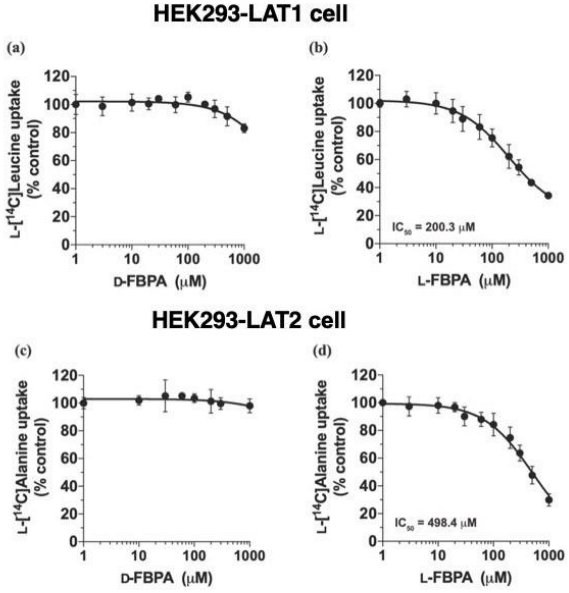

Figure 1. In vitro inhibition experiments. (a,b) HEK293-LAT1 cells and (c,d) HEK293-LAT2 cells. [^{14}C] leucine and [^{14}C] alanine uptakes via LATs were inhibited by increasing the non-radiolabelled compound in the case of L-FBPA. However, D-FBPA showed no significant inhibitory effect in HEK293-LAT1 or HEK293-LAT2 cells

Next, we examined the whole-body distribution of D-[^{18}F]FBPA *in vivo*. Figure 2a shows TACs of normal organs. The accumulation in the kidney was dramatically faster and higher, showing its peak at approximately 11 to 13 minutes after injection. On the other hand, accumulation in the liver, brain, heart, lungs, muscle, and pancreas remained very low throughout the PET imaging duration. Figure 2b exhibits SUV_{mean} at 60 minutes after injection. Significantly higher accumulation in the kidney was observed.

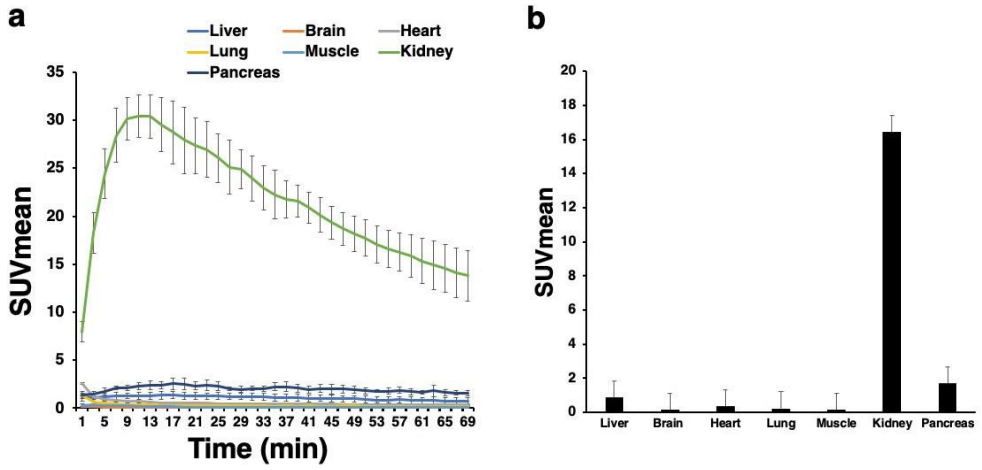

Figure 2. Whole-body distribution of D-[^{18}F]FBPA. (a) Time activity curves and (b)SUV_{mean} at 60min after administration of D-[^{18}F]FBPA in normal organs were compared. Rapid excretion from the kidneys was observed

To investigate whether the accumulation of D-[^{18}F]FBPA is observed specifically in the tumor, we compared its uptake in C6 glioma and inflammatory lesion. PET images of D-[^{18}F]FBPA in these two tissues are shown in Figure 3a. Faint uptake, which is the same uptake level as background, was observed in the C6 glioma, as well as the inflammatory lesion. TACs of D-[^{18}F]FBPA showed similar courses with a gradually decreasing trend in the C6 glioma and inflammatory lesion (Figure 3b). Static PET analysis showed low accumulation of D-[^{18}F]FBPA both in the C6 glioma and inflammatory lesion although there was a statistical difference between glioma and inflammation (SUV_{max}= 0.80±0.16 and 0.56±0.09; T/M ratio=8.8±2.7 and 5.6±2.2, respectively) (Figure 3c,d).

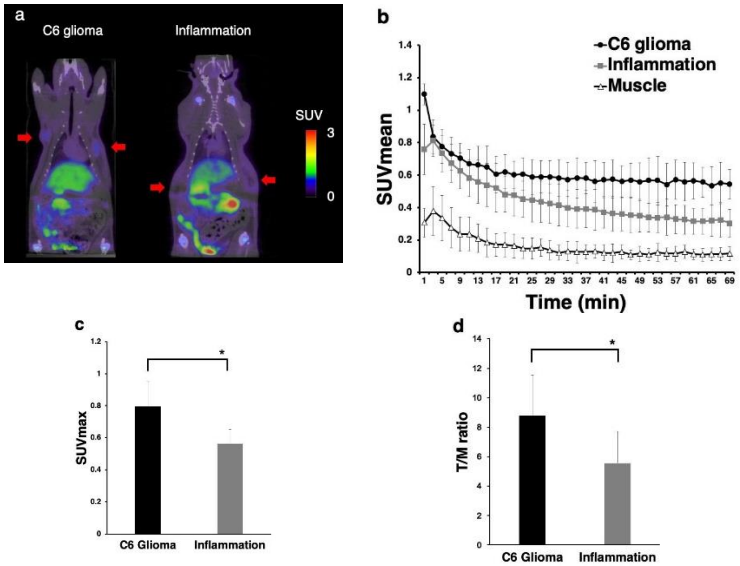

Figure 3. (a) D-[^{18}F]FBPA PET images of xenograft models of C6 glioma and subcutaneous inflammation model. Arrows indicate the bilateral tumors or inflammatory lesions with low uptakes. Comparison of (b) Time activity curves, (c) SUV_{max} and (d) T/M ratio between C6 glioma and inflammatory lesion in D-[^{18}F]FBPA PET.

Lastly, we histologically confirmed C6 glioma and inflammation tissue in each rat model. Irregular tumor cells in the C6 glioma and neutrophil accumulation surrounding the turpentine oil in the inflammatory lesion was observed in a rat xenograft model and inflammation model, respectively (Figure 4)

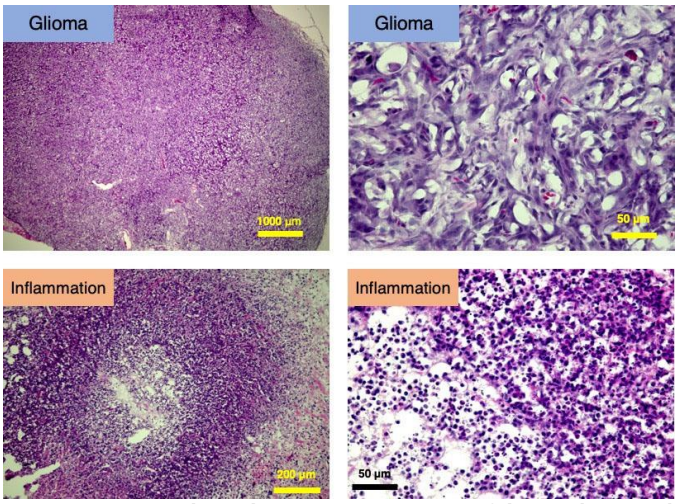

Figure 4. Hematoxylin and eosin staining images of the C6 glioma and inflammatory lesion (low and high magnification). C6 glioma showed atypical cells of irregular size in the nucleus. Inflammatory lesion showed neutrophil accumulation surrounding the turpentine oil

Discussion

In the present study, we evaluated the utility of D-[^{18}F]FBPA as a tumor-specific probe using the xenograft and inflammation models. As a result, D-[^{18}F]FBPA PET showed very low uptakes both in C6 glioma and inflammatory lesion. It was very difficult to differentiate tumor tissue from non-tumor tissue such as inflammatory lesion in D-[^{18}F]FBPA PET. C6 glioma was reported to show a high expression of LAT1/CD98 heterodimer with high blood flow (15). Since C6 glioma is a representative tumor with a functional expression of LAT1, we previously selected C6 glioma to evaluate the tumor imaging capability of L-[^{18}F]FBPA as a LAT1 PET probe (3). In the present study, we used C6 glioma as well so that we could compare the results from the previous study using L-[^{18}F]FBPA and the present study, D-[^{18}F]FBPA.
Our group previously reported that L-[^{18}F]FBPA is LAT1-selectively transported and shows specific accumulation in the tumor (3). In that study, we used the same method as the present study to evaluate the accumulation of L-[^{18}F]FBPA in vivo and demonstrated that SUV_{max} of L-[^{18}F]FBPA in C6 glioma and inflammation was 3.23±0.40 and 1.86±0.19, respectively. On the other hand, the present study showed that the accumulation of D-[^{18}F]FBPA was 0.80±0.16 in C6 glioma and 0.56±0.09 in inflammation (Figure 3c), which is significantly lower than that of L-[^{18}F]FBPA (Supplemental Figure). This comparison between L- and D-[^{18}F]FBPA revealed that L-[^{18}F]FBPA showed substantially larger SUV_{max} difference between glioma and inflammation. Therefore, L-[^{18}F]FBPA has a greater advantage than D-[^{18}F]FBPA in distinguishing C6 glioma from the inflammatory lesion in PET imaging. A series of our studies strongly suggested that L-[^{18}F]FBPA is a superior PET probe for tumor diagnosis than D-[^{18}F]FBPA .

Moreover, D-[^{18}F]FBPA can also have some disadvantages in BNCT. Indeed, D-[^{18}F]FBPA showed a high T/M ratio (Figure 3d) in the present study as well as Kanazawa et al. reported (12). However, because the absolute concentration of D-[^{18}F]FBPA was significantly smaller than that of L-[^{18}F]FBPA, it is necessary to investigate whether BNCT using D-[^{18}F]FBPA/D-BPA can achieve a better therapeutic effect than L-[^{18}F]FBPA/L-BPA. Besides, the previous study reported that the racemization was observed in the Cu-mediated radiofluorination process of 2-[^{18}F]Fluorophenylalanine (16). The present study suggested that the racemization of L-/ D-[^{18}F]FBPA should be minimized in the L-[^{18}F]FBPA synthesis process, especially for the future development of L-[^{18}F]FBPA using F^-, to maintain the sufficient accumulation of [^{18}F]FBPA in the target.

In order to reveal the cause of this different kinetic between L- and D-[^{18}F]FBPA, we performed both *in vitro* and *in vivo* studies. As indicated in the *in vitro* inhibition study, it is

speculated that D-FBPA was not a substrate of LAT1 and LAT2 because of its low inhibitory effect. In the case of the *in vivo* whole-body distribution PET analysis, significantly low uptake of D-[^{18}F]FBPA was observed in the liver, brain, heart, lung, muscle, and pancreas, along with the rapid excretion from the kidney. It was reported that LAT2 is expressed in the normal organ (8). Therefore, this result can be attributed to a low affinity of D-[^{18}F]FBPA for LAT2, although it is possible that D-[^{18}F]FBPA also shows low affinity for other amino acid transporters expressed on the normal organ. On the contrary, LAT1 is known to be highly upregulated in the malignant tumor (4). *In vivo* D-[^{18}F]FBPA PET showed lower uptakes in glioma compared with L-[^{18}F]FBPA, reflecting the lower transporter affinity to LAT1. However, since L-BPA is reported to be transported through other transporters than LAT1 such as $ATB^{0,+}$ (17), there is a possibility that D-[^{18}F]FBPA is transported through other amino acid transporters. However, its effect is thought to be, if any, very small based on the fact that the absolute uptake of D-[^{18}F]FBPA in C6 glioma was significantly low.

To the best of our knowledge, this is the first study to investigate the affinity of D-[^{18}F]FBPA for LAT and its effect on PET image. However, some limitations should be noted. First, since we used a single C6 glioma model in the present study, the behavior of D-[^{18}F]FBPA should be examined using other types of cancer cells to make a more generalized conclusion. Besides, although it was suggested that D-[^{18}F]FBPA was not a substrate of LAT1 and LAT2 in *in vitro* inhibition study, a faint uptake of D-[^{18}F]FBPA in the tumor was observed on the PET imaging. Therefore, we should investigate in further research whether there is another transport mechanism of D-[^{18}F]FBPA into C6 glioma and normal tissue.

Conclusion

In vitro inhibition experiment showed that D-[^{18}F]FBPA is not a LAT1-specific substrate. Besides, *in vivo* PET analysis demonstrated that it is difficult to distinguish C6 glioma from inflammation tissue using D-[^{18}F]FBPA, suggesting that D-[^{18}F]FBPA is not suitable as a tumor diagnosis PET probe compared to L-[^{18}F]FBPA.

Acknowledgments

We would like to thank Takanori Kobayashi for his excellent technical assistance and support. D-BPA and D-[^{18}F]FBPA was kindly gifted from Prof. Mitsunori Kirihata (Osaka Prefectural University).

Funding

This study was funded by the KAKENHI Grant-in-Aid for Scientific Research (S) (Number 24229008) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and the Quantum Innovation for Safe and Smart Society (QiSS) program on the open innovation platform with enterprises, research institute and academia (OPERA) from the Japan Science and Technology Agency (JST), Japan.

Compliance with ethical standards

Conflict of Interest

The authors have no potential conflict of interest relevant to this article.

Ethical approval

All animal experiments were performed under the guidelines of the Institute of Experimental Animal Sciences. The protocol was approved by the Animal Care and Use Committee of the Osaka University Graduate School of Medicine.

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