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# $^{18}$F-Fluoroglucosylation of an arginine-arginine-leucine peptide as a potential tumor imaging agent for positron emission tomography

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► ***Original article***

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## ABSTRACT

***Background:*** Based on the principle of oxime formation, $^{18}$F labeling of polypeptides can be achieved via a reaction between an aldehyde group-containing $^{18}$F-prosthetic group and an aminooxy-modified polypeptide. The focus of this study was to investigate the one-step synthesis of 2-[$^{18}$F] fluoro-2-deoxyglucose ($^{18}$F-FDG)- arginine-arginine-leucine (RRL) from open-ring $^{18}$F-FDG and the aminooxy-modified RRL peptide cyclo(RRLfK)-$ONH_2$ and to study the biological distribution of $^{18}$F-FDG-RRL in a nude mouse model of human neuroglioma. ***Materials and Methods:*** The aminooxy-modified RRL peptide cyclo(RRLfK)-$ONH_2$ was used as the precursor to react with $^{18}$F-FDG at 100 °C and different pH values for 30 minutes to synthesize $^{18}$F-FDG-RRL. The labeling yield, radiochemical purity, and *in-vitro* stability of the product were measured, and the biological distribution of $^{18}$F-FDG-RRL in tumor-bearing nude mice was analyzed at 30 minutes, 60 minutes and 120 minutes. ***Results:*** The labeling yield of $^{18}$F-FDG-RRL was (25.5±5.0) % at a pH of 2.0, and its radiochemical purity was greater than 95%. $^{18}$F-FDG-RRL was mainly excreted through the kidneys, with rapid blood clearance. One hour after injection, the uptake of $^{18}$F-FDG-RRL in tumors was (1.83±0.12) injected dose per gram of tissue (%ID/g), with a tumor/muscle ratio of 7.03±0.04, a tumor/blood ratio of 4.36±0.21 and a tumor/brain ratio of 7.53±1.37. ***Conclusion:*** The synthesis of $^{18}$F-FDG-RRL can be achieved through oximation. This method is straightforward and easy to promote. $^{18}$F-FDG-RRL has rapid blood clearance and high uptake by tumors.

***Keywords:*** *Oxime, 2-fluoro-2-deoxyglucose, arginine-arginine-leucine, peptide.*

## INTRODUCTION

2-Fluoro-2-deoxy-D-glucose ($^{18}$F-FDG) is currently the most commonly used imaging agent in positron emission tomography (PET) [1]. Its molecular structure is similar to that of glucose, so modifications similar to glycosylation can be achieved through [$^{18}$F] labeling with $^{18}$F-FDG as a prosthetic group. $^{18}$F-FDG undergoes ring opening at 100°C and exists in both the cyclic and linear states. The aldehyde group at position 1 of linear $^{18}$F-FDG undergoes an oximation reaction with an aminooxy group under acidic conditions to achieve $^{18}$F labeling of polypeptides [2-7].

In 2008, Wuest *et al.* first reported the preparation of $^{18}$F labeled tracers based on $^{18}$F-FDG [6] in the production of $^{18}$F-FDG-maleimide-hexyloxime ($^{18}$F-FDG-MHO) by linking $^{18}$F-FDG to aminooxy-functionalized maleimide, with a yield of approximately 45%-69%. The direct labeling of aminooxy-modified peptides with $^{18}$F-FDG was reported by Namavari *et al.* [5] and Hultsch *et al.* [8]. Namavari *et al.* directly labeled linear and circular arginine-glycine-aspartic acid (RGD)

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modified with an aminooxyacetyl group using $^{18}$F-FDG. The reaction was carried out at 100°C and pH 1.5-2.5 for 60 minutes, and it had a yield of approximately 41.4%. Hultsch *et al.* used $^{18}$F-FDG to label an aminooxy-modified RGD cyclic peptide in a reaction carried out at 120°C and pH 2.5 for 20 minutes, and it had a yield of approximately 56-93%. Wuest *et al.* [9] also used $^{18}$F-FDG to label the monomer, dimer and tetramer of an aminooxy-modified neurotensin (8-13) fragment (NT (8-13)). The labeling yield of the product from the monomer was the highest (80%). Wuest *et al.* also believed that the substituted fluoro group at the α-position of the carbonyl group of open-ring FDG was likely to increase the carbonyl reactivity, which made it easier for the aminooxy group to react with $^{18}$F-FDG than with glucose. Simpson *et al.* [10] used $^{18}$F-FDG to label aminooxy-modified and PEGylated biotin derivatives, with a product labeling yield of up to 100% and low specific activity. Jammaz *et al.* [11] employed $^{18}$F-FDG to label aminooxy-modified folic acid and methotrexate, with a labeling yield greater than 80% and a radiochemical purity greater than 98%.

Peptides could find important applications in targeting tumors and are good candidates because of their characteristic properties [12, 13]. Many studies have shown that the peptide arginine-arginine-leucine (RRL) can target tumor vascular endothelial cells and tumor parenchyma cells [14, 15]. In this study, $^{18}$F-FDG was used as a prosthetic group to undergo a condensation reaction with an aminooxy-modified RRL peptide to synthesize $^{18}$F-FDG-RRL in one step. The $^{18}$F-FDG-RRL was evaluated for its *in-vitro* stability and biodistribution. The RRL peptide was labeled with $^{18}$F for the first time, and the $^{18}$F-labeled RRL peptide was expected to be a potential PET imaging agent for tumor targeting.

## MATERIALS AND METHODS

### *Peptide synthesis and modification*

The RRL peptide sequence cyclo(RRLfK) was designed and subjected to aminooxy modification to obtain cyclo(RRLfK)-$ONH_2$. The aminooxy group was attached to the side chain of the lysine residue. Cyclo(RRLfK)-$ONH_2$ was synthesized using solid-phase peptide synthesis followed by manual conjugation of Bis-Boc-aminooxyacetic acid with subsequent deprotection(Shanghai Science Peptide Biological Technology Co., Ltd., China).

Peptides were purified via reversed-phase high performance liquid chromatography (RP-HPLC) with an Inertsil ODS-SP HPLC column (5μm 4.6×250mm) (Shimadzu Corporation, Japan) eluted with a gradient from 98 to 0% solvent A (0.1% trifluoroacetic in 100% water) and 2–100% solvent B (0.1% trifluoroacetic in 100% acetonitrile) at 1.0 ml/minute, with monitoring at 214 nm. The purity of the peptide was determined by RP-HPLC as listed, and its identity was confirmed via mass spectral analysis (Shimadzu Corporation, Japan).

### *Radiolabeling*

The preparation of 120 μL of $^{18}$F-FDG (HTA Co., Ltd. , China) solution was carried out with the following specifications: $^{18}$F-FDG 4-6mCi, 16% aqueous ethanol-saline solution and 0.4% trifluoroacetic acid (TFA) (Tokyo Chemical Industry Co., Ltd., Japan). To the above solution, 2 mg of cyclo(RRLfK)-$ONH_2$ was added. The pH was adjusted to 2.0,3.0,4.0 and 5.0, respectively. The mixture was kept at 100°C for 30 minutes (figure 1).

The labeling yield and radiochemical purity of the labeled product $^{18}$F-FDG-c (RRLfK) were determined using RP-HPLC. Gradient elution was performed as follows: 0-3 minutes, 5% B; 3-33 minutes, 5%-65% B; 33-36 minutes, 65%-90% B; 36-39 minutes, 90% B; 39-42 minutes, 90%-5% B (buffer A = 0.1% TFA in acetonitrile, buffer B = 0.1% aqueous TFA solution). The flow rate was 1 mL/minute.

### *Determination of in-vitro stability*

$^{18}$F-FDG-RRL (100 μL, 3.7 MBq) was mixed well with 1 mL of fresh human serum and incubated at 37°C. The radiochemical purity was measured at 30, 60, 90, 120, 180, 240, 300 and 360 minutes by radio-HPLC (LabAlliance Company, USA).

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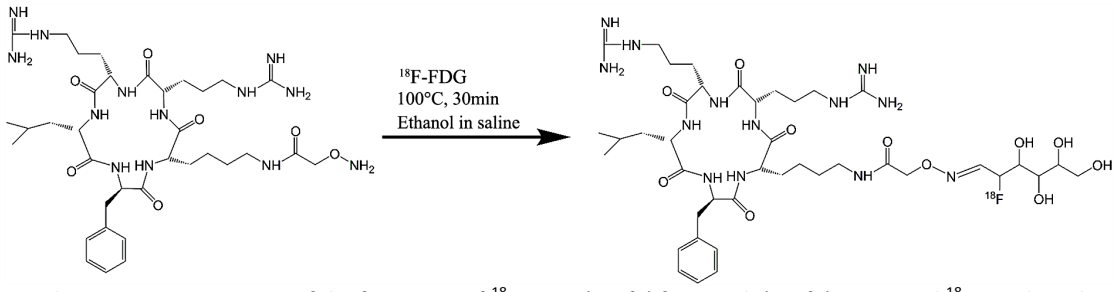



**Figure 1.** Schematic representation of the formation of $^{18}$F-FDG-c(RRLfK) from cyclo(RRLfK)-ONH2 and $^{18}$F-FDG based on oxime formation at 100°C for 30 min.

### *Examination of biodistribution*

The human malignant glioblastoma cell line U87MG was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Twenty male BALB/c nude mice, approximately 4-6 weeks of age and weighing 16 -20 g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The animal experiments were approved by the Animal Welfare and Ethics Committee of Qingdao University and strictly followed the standards in the "Guide for the Care and Use of Laboratory Animals" published by National Academies Press (US). A 0.2-mL single cell suspension ($1\times10^6$ cells/mL on average) was injected subcutaneously into the right shoulder of the mice, and the long axis (a) and short axis (b) of the tumors were measured during tumor growth using a Vernier caliper. The tumor volume was calculated based on the formula $a\times b^2/2$. The biodistribution was examined when the tumor volume reached approximately 1.0 $cm^3$.

The $^{18}$F-FDG-RRL solution (0.2 mL, 0.74 MBq) was injected via the tail vein into 9 tumor-bearing nude mice. At 30, 60 and 120 minutes after injection, the mice were euthanized via $CO_2$ inhalation. Samples of the blood, tumor, brain, heart, lungs, stomach, liver, intestines, kidneys and bone were collected, and the injected dose per gram of tissue (%ID/g) was calculated for each sample type. In addition, the tumor/blood, tumor/muscle and tumor/ brain ratios were calculated.

### *Statistical analysis*

Statistical analysis was performed using SPSS 18.0 software. A *t-test* and one-way analysis of variance were employed. Data measurements are expressed as ($\bar{x}$ ± s). One-way analysis of variance (ANOVA) was used for comparisons of variables. $P<0.05$ was considered statistically significant.

## RESULTS

### *Peptide synthesis and modification*

Cyclo(RRLfK)-$ONH_2$ was successfully synthesized using solid-phase peptide synthesis followed by the manual conjugation of bis-Boc-aminooxyacetic acid with subsequent deprotection. HPLC analysis of the product indicated the formation of several compounds consisting of a major product (95.163%, retention time of 18.582 minutes) accompanied by at least 4 smaller peaks at retention times of 17.864, 18.361, 18.934 and 19.139 minutes (figure 2A). The mass spectrometry (ESI-MS) analysis of cyclo(RRLfK)-$ONH_2$ showed a mass peak with a m/z of 387.98 ([M+2H]2H+)(figure 2B).The experimentally observed molecular weight(773.96) correlated well with the theoretical molecular weight(773.94).

### *Radiolabeling*

$^{18}$F-FDG-RRL was prepared from cyclo (RRLfK)-$ONH_2$ and $^{18}$F-FDG in a one-step method based on the mechanism of oximation. The reaction process is simple and easy to automate. The average labeling yields of three consecutive $^{18}$F-FDG-RRL syntheses at different pH values (2.0, 3.0, 4.0 and 5.0) were (25.5±5.0) %, (20.0±5.0) %, (20.0±4.0) %, and (2.5±2.0) %, respectively (figure 3). The radiochemical purity was above 95%.

$^{18}$F-FDG-RRL was purified by HPLC, and the



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radioactive trace (retention time of approximately 15.2 minutes) is basically consistent with the ultraviolet (UV) trace (retention time of approximately 14.2 minutes) (figure 4).

### *Analysis of in-vitro stability*

The radiochemical purity was (96.1±0.39) %, (95.9±0.31) %, (95.5±1.38) %, (94.9±0.34) %, (93.9±0.61) %, (93.0±0.35) %, (91.9±0.46) % and (90.1±0.51) % at 30, 60, 90, 120, 180, 240, 300 and 360 minutes, respectively (figure 5).

### *In-vivo biodistribution*

The biodistribution of $^{18}$F-FDG-RRL was measured in U87MG xenograft-bearing nude mice 2 hours after intravenous injection of the probe (table 1). $^{18}$F-FDG-RRL rapidly accumulated in the tumors, and its radioactivity distribution was (2.74±0.25) % ID/g, (1.83±0.12) % ID/g and (1.24±0.16) % ID/g at 30, 60 and 120 minutes after injection, respectively. The radioactive distribution of $^{18}$F-FDG-RRL was high in the kidney, at (3.89±0.24) % ID/g, (1.95±0.15) % ID/g and (1.12±0.43) % ID/g at 30, 60 and 120 minutes after injection, respectively. $^{18}$F-FDG-RRL showed lower levels of radioactivity distribution in other tissues. The tumor/blood, tumor/muscle and tumor/brain ratios were relatively high and increased with time. The tumor/blood ratios were 3.19±0.04, 4.36±0.21 and 8.26±0.32 at 30, 60 and 120 minutes after injection, respectively; the tumor/muscle ratios were 5.07±0.10, 7.03±0.04 and 8.70±0.05, respectively; and the tumor/brain ratios were 5.71±0.08, 7.53±1.37 and 8.07±0.56, respectively (figure 6).

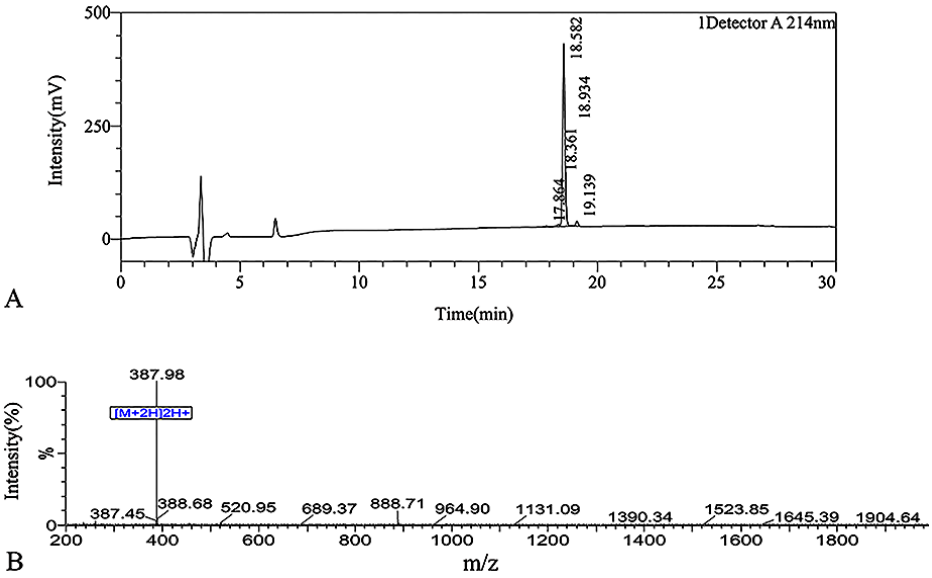



**Figure 2.** HPLC (A) and MS (B) analysis of cyclo(RRLfK)-ONH2. HPLC analysis showed a major product (95.163%, retention time of 18.582 min) accompanied by 4 smaller peaks at retention times of 17.864, 18.361, 18.934 and 19.139 min. The MS analysis showed a mass peak with a m/z of 387.98 ([M+2H]2H+).

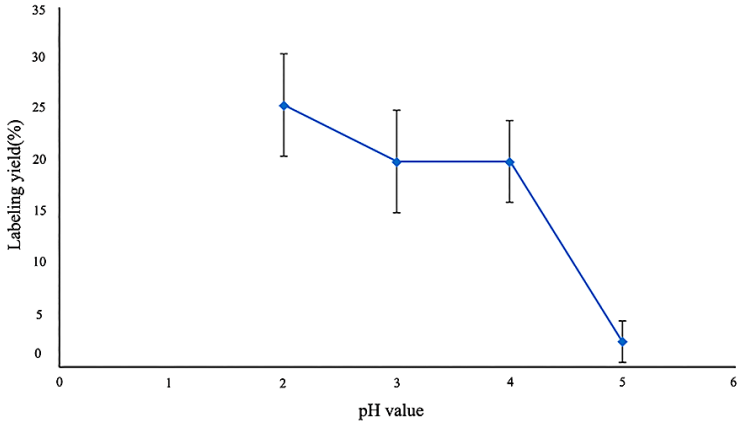



**Figure 3.** Labeling yield of $^{18}$F-FDG-RRL synthesized at different pH values. The labeling yield decreased significantly at pH 5.

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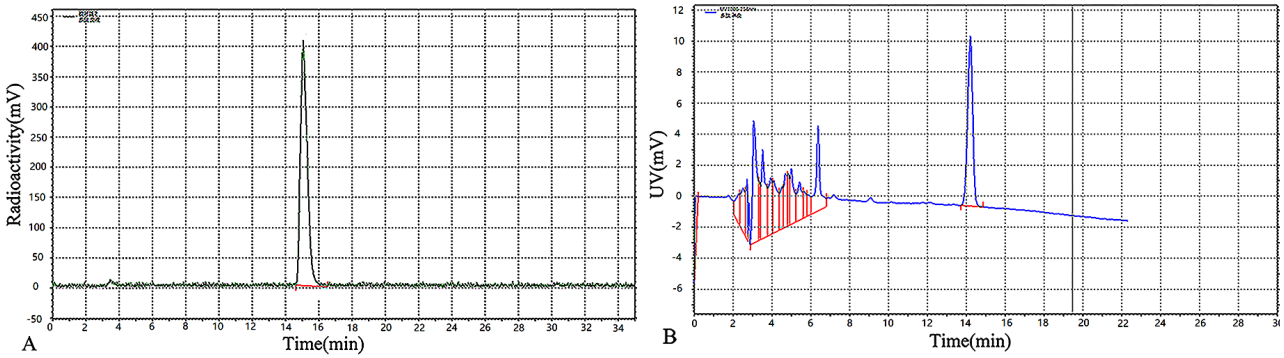



**Figure 4.** Analytical HPLC profiles of 18F-FDG-RRL after radio-HPLC. A is the radioactive trace, and B is the UV trace. The radioactive trace (retention time of approximately 15.2 min) is consistent with the ultraviolet (UV) trace (retention time of approximately 14.2 min).

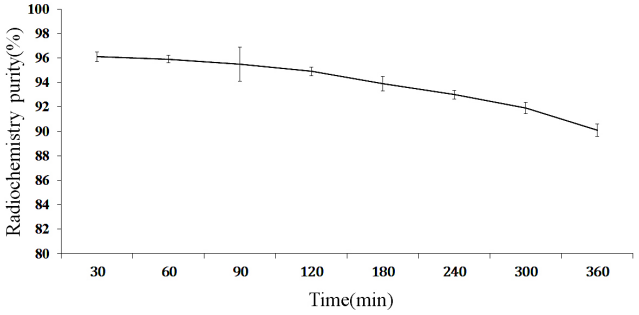



**Figure 5.** In-vitro stability of $^{18}$F-FDG-RRL. Means with error bars for radiochemical purity at different times; n=5. The bars show the standard deviation (SD).

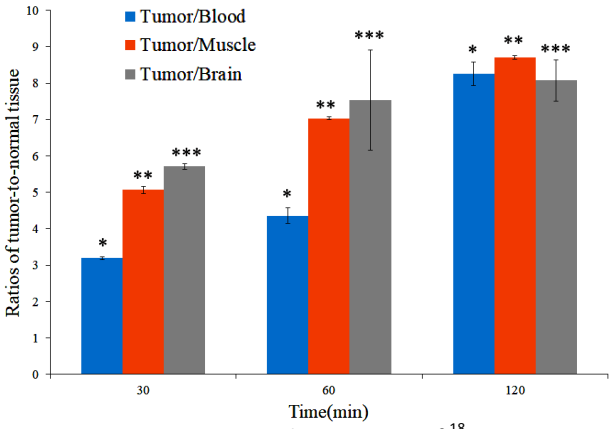



**Figure 6.** Tumor-to-normal tissue ratios of $^{18}$F-FDG-RRL. Means with error bars for tumor/background; n=5. The bars show the standard deviation (SD). Tumor/background increased with time (*p<0.001, **p<0.001, ***p<0.05).

## DISCUSSION

$^{18}$F-FDG is currently the most widely used positron imaging agent. The advantages of $^{18}$F labeling with $^{18}$F-FDG as a prosthetic group are that (1) $^{18}$F-FDG is low-cost and easy to obtain, (2) the synthesis method is a simple one-pot reaction, (3) $^{18}$F-FDG has a molecular structure similar to a monosaccharide and (4) $^{18}$F labeling with $^{18}$F-FDG as a prosthetic group can achieve effects similar to glycosylation modification. The disadvantages are that (1) $^{18}$F-FDG in clinical use contains excessive glucose and needs to be purified prior to labeling [8], (2) the labeled molecules must be specifically modified and (3) the large steric hindrance from the glycosyl group may affect the affinity of the labeled product for the target [16].

The highest labeling yield of this reaction was (25.5±5.0) % at pH 2.0, and as the pH increased, the labeling yield decreased to only (2.5 ± 2.0) % at pH 5.0, which is consistent with the study by Namavari *et al.* [5], which showed that the optimal pH of this reaction was 1.5-2.5 and that nearly no product was obtained when the pH was above 4. The possible reasons are as follows: when the pH is in the range of 1.5-2.5, (1) the pyran ring of $^{18}$F-FDG is easier to open, exposing the aldehyde group, and (2) the carbon atom of the aldehyde group of $^{18}$F-FDG is positively charged due to protonation and more susceptible to a nucleophilic substitution reaction with the aminooxy group of the polypeptide. The labeling yield may be further improved through $^{18}$F-FDG purification, RRL peptide modification and optimization of the

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reaction time and temperature of the product separation. It is worth noting that clinically applied $^{18}$F-FDG will produce excessive glucose during production and that the presence of glucose will inhibit the labeling reaction. Consequently, further purification of $^{18}$F FDG is required to separate the glucose before the labeling reaction [8, 9]. The radiochemical purity of $^{18}$F-FDG-RRL in human serum was still above 90% after 6 hours, which meets the requirements for imaging.

The results of the *in-vivo* biodistribution study showed that the radioactivity uptake was highest in the kidney, suggesting that $^{18}$F-FDG-RRL is mainly excreted through the kidneys. This is likely due to the increased hydrophilicity of the RRL peptide when it is linked to $^{18}$F-FDG, which makes it easy to excrete through the kidneys, thereby reducing the background. $^{18}$F-FDG-RRL showed low levels of uptake in brain tissue, with a high tumor/brain ratio, which suggests that this imaging agent is suitable for PET imaging of brain tumors, with great advantages over the other commonly used PET imaging agent, $^{18}$F-FDG, for brain tumor imaging. The uptake of $^{18}$F-FDG-RRL was high in tumors, with increasing tumor/muscle, tumor/blood and tumor/brain ratios over time, which suggests that $^{18}$F-FDG-RRL is highly valuable for PET imaging of tumors.

## CONCLUSION

In summary, $^{18}$F labeling of polypeptides with $^{18}$F-FDG as a prosthetic group is a feasible method that is simple, convenient and cost-effective. This method also improves the hydrophilicity of the labeled peptides and facilitates the clearance of the tracing agent from nontarget tissues. Further improvement of the labeling yield and reduction of the reaction time and number of precursors required will be the focus of future research.

***Conflicts of interest*:** *Declared none.*

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