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A ferritin-containing nanoconjugate as MRI image-guidance to target Necl-5, a tumor-surface antigen: a potential thermal accelerant for microwave ablation

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ABSTRACTS

Purpose: A ferritin-containing nanoparticle conjugated with a target-specific antibody was investigated as a MRI contrast agent for tumor detection. A genetically modified ferritin to markedly improve Fe (III) payload (up to 7,000 Fe ions), was chemically tethered to a monoclonal antibody against rat Nectin-like molecule 5 (Necl-5). Necl-5 is a cell surface glycoprotein that is highly expressed on the cell surface of many common epithelial cancers, including prostate cancer. It was previously demonstrated that this novel nanoconjugate agent exhibited effective in vitro targeting of Necl-5 expressing tumor cells and exhibited strong MRI contrast characteristics via shortening of T₂. Here, we demonstrate that the nanoconjugate-Necl-5 interaction can be exploited to target and detect tumor in vivo by MRI.

Procedure: Using an in vivo tumor model (i.e., tumor size 0.5-1 cm, immunodeficient beige/nude/xid mouse, xenograft injection with transformed rat prostate cells), efficacy of the conjugate targeting the tumor was examined. We used two injection strategies, a direct and a tail vein injection (0.8 mg, 300 μ L per subject). Pre-injection baseline and post-injection scans were performed with the following spin-echo sequence parameters: Field of view = 90x53mm, reconstruction matrix size = 192x114, slice thickness = 1mm (10 slices), repetition time (T_R) = 2070 ms, echo times (T_E) = 11-198 ms in 11ms steps (18 echoes), number of averages = 2, acquisition time per scan = 7min 56s.

Results: All T₂ data obtained were converted to R₂ for demonstration purposes ($R_2 = 1/T_2$). The tail vein injected conjugate significantly increased R₂ response ($22.9 \pm 5.2 \text{ s}^{-1}$) as compared to control ($13.5 \pm 1.7 \text{ s}^{-1}$) at 4 h. The weaker R₂ increase was noted ($15.2 \pm 2.0 \text{ s}^{-1}$) at 24 h. No notable changes in R₂ were observed in surrounding tissues regardless the stages of the measurement. We also measured the initial conjugate kinetics for both injection methods with respect to the ability of targeting the tumor. Direct injection of the nanoconjugate in to the center of the tumor showed a stronger and more rapid increase in R₂ than the tail vein injection.

Conclusion: The nanoconjugate interacts strongly and selectively in situ with Necl-5 overexpressing tumor cells. Direct injection of the nanoconjugate into the body of the tumor caused a more significant in situ R₂ increase in MRI than the tail vein injection. Varying degrees of R₂ increase within the tumor mass is likely to represent different distribution patterns of the conjugate, reflective of tumor heterogeneity.

Key Words: Ferritin, nanoconjugate, Necl-5, monoclonal antibody, Xenograft, T₁ and T₂ (R₁ and R₂), electromagnetic field, self-assembly, microwave energy

INTRODUCTION

According to the American Cancer Society, it is estimated that in 2016 more than 1.6 million new cancer cases will be diagnosed and approximately 595,000 Americans are expected to die of cancer [1]. Cancer remains the second most common cause of death in the US, accounting for 1 in every 4 deaths. Despite recent advances in medical research and development, effective diagnosis and improved cancer treatment options remain elusive. In order to overcome limitations for the lack of early detection methods and/or selective tumor-targeting therapeutic options, current paradigm for cancer research places an emphasis on the discovery of improved tumor-specific biomarkers, development of increasingly sensitive detection/visualization methods for accurately assessing treatment options and the selective delivery of anti-tumor agents to primary and secondary metastatic tumors.

Nanoparticles provide unique approaches for cancer detection and treatment [2]. Multimodality of various nanoparticles for magnetic resonance imaging (MRI), drug delivery, specific targeting and thermal ablation of tumor cells have been proposed by the biomedical community for several years [3-6]. While the most common MRI contrast agents with the close relevance to the multimodality currently in use are the superparamagnetic iron oxide nanoparticles improvement of these agents in the clinical setting is strongly desired [7].

Ferritin, which plays a role in the storage and maintenance of iron homeostasis in the cell has been investigated as a MRI contrast agent [8-13]. Although natural ferritin has relaxivity values too low to act as an effective contrast agent genetically modified forms that encapsulate more iron than natural forms have been developed with significantly improved MRI contrast characteristics [11]. One such form, the genetically engineered ferritin cage derived from *Archaeoglobus fulgidus* encapsulates up to 7,000 iron (III) ions and has demonstrated strong MRI contrast characteristics with approximately two-orders greater relaxivity than endogenous ferritin [12,13].

Nectin-like molecule 5 (Nect-5) is a cell surface glycoprotein belonging to the Nectin family of cell adhesion molecules [14]. Nect-5 is involved not only in cell-cell adhesion, but also in cell migration and proliferation particularly during fetal development. Once the fetal development phase is over, the Nect-5 activity becomes dormant to the extent that Nect-5 is nearly undetectable. Interestingly, Nect-5 is highly expressed in many carcinomas, common cancers originated from epithelial cells [15-19]. This strong constitutive expression of Nect-5 by the transformed rat epithelial cells made this cell model an especially attractive target biomarker for the development of our nanoconjugate tumor detection strategy. Furthermore, it is noteworthy that epithelial cancer cells in humans are overexpressed with CD-155, an ortholog of rat Nect-5 (also known as poliovirus receptor (PVR)) that is involved in cell migration, invasion, proliferation as well as metastasis [20]. This suggests that the use of Nect-5 as a target biomarker in our model can be well suited for studying human epithelial cancers by using CD-155.

We have previously showed in vitro that the Nect-5/ferritin nanoconjugate targeted transformed Nect-5 expressing rat prostate epithelial cells with high specificity and exhibited strong T_2 MRI contrast characteristics [21]. In the present study, we demonstrate specific in situ targeting of the tumor and show effective utility of the nanoconjugate as an effective T_2 MRI contrast agent.

MATERIALS AND METHODS

Cell Culture and Tumor Model

The origin and isolation of the rat prostate epithelial cells has been described [25,26] The development and characterization of the transformed rat prostate epithelial cells used in this study will be described elsewhere (manuscript in preparation). Briefly, the rat prostate epithelial cells were cultured in a 1:1 mixture of RPMI 1640 (Gibco, Carlsbad, CA) and MCDB 153 (Sigma-Aldrich, St. Louis, MO) supplemented with sodium bicarbonate (1.9 g/L), sodium pyruvate (0.5%), fetal bovine serum (FBS) (5%, Hyclone, Logan, UT), epidermal growth factor (0.02 μ g/ml, BD Biosciences, San Jose, CA), bovine pituitary extract (5 μ g/ml, BD Biosciences), dexamethasone (2 mM in 95% EtOH), glutamine (1%), gentamycin (0.1 mg/ml, Gibco), ITS (Liquid Media Supplement, 0.25%, BD Biosciences), forskolin (2.5 μ g/ml, Calbiochem, San Diego, CA) and Normocin and incubated at 37°C in a 5% CO₂ humidified atmosphere. Cells were grown to approximately 75-80% confluence trypsinized before being harvested for injections into mice.

All animal protocols described in these studies were approved by the Brown University and Rhode Island Hospital

Institutional Animal Care and Use Committees (IACUC). The tumor model used in this study has been previously described [27]. Briefly, cells for injection were harvested with trypsin/EGTA when they reached approximately 80% confluence. Cells were washed and suspended in sterile HBSS. 10×10^6 cells were injected into the left rear flank of 4-6 week old female beige/nude/xid triple deficient mice. Injected mice were closely monitored for tumor growth for 10-12 days. Nanoconjugate agent, prepared as described below, was injected 10-12 days post injection of cells via the tail vein or direct injection into the tumor mass.

Immunofluorescence Assay and Digital Imaging

Tumor and liver tissues were harvested from euthanized mice 4 h after MR imaging of the tail vein injection study. Each tissue type was collected separately either from control (ferritin only) or from the conjugate. Frozen sections were cut at 20 microns thickness. Slides were dried flat, and stored in slide boxes at -80°C until ready to be stained. Slides were rinsed briefly in FTA buffer, pH 7.2. Secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody, Life Technologies) was diluted 1:400 in the same buffer, and applied to tissue sections while slides lay flat. Approximately 0.1 ml of antibody was applied to each tumor section, and approximately 1.8 ml to each of the larger liver sections. Slides were incubated in a humidified chamber, protected from light, at room temperature, for 45 minutes, then placed in a staining rack and rinsed with 3 changes of FTA buffer, 2 minutes each, and transferred to a fresh change of FTA buffer. Sections were cover-slipped with VectaShield mounting medium containing DAPI used as a counter-stain for contrast. Confocal images were acquired with a Nikon C1si confocal (Nikon Inc. Mellville NY) using diode lasers 402 and 488. Serial optical sections were performed with EZ-C1 computer software (Nikon Inc. Mellville, NY). Each wavelength was acquired separately by invoking frame lambda. Z series sections were collected at $0.3\ \mu\text{m}$ with a 40x Plan Apo lens. Deconvolution and projections were performed in Elements (Nikon Inc. Mellville, NY) computer software.

Nanoconjugate

The enrichment of modified ferritin with iron (III) ions, conjugation of the iron-enriched ferritin with monoclonal antibody to rat Necl-5 and in vitro demonstration of the utility of the ferritin nanoconjugate as a novel MRI contrast agent has recently been described [21]. Briefly, we have shown that the ferritin nanoconjugate can be used as a contrast agent for T_2 and T_2^* (susceptibility) MR image contrast weightings from which contrast changes are distinguishable at dose concentrations of $50\ \mu\text{g/ml}$. We also measured the relaxivity (rate of MR signal decay per unit concentration of agent) of the ferritin nanoconjugate and found it to be on the order of 100-times greater than that of endogenous ferritin.

MR Imaging

Mice were imaged using a Siemens Tim Trio 3 Tesla scanner. Two anesthetized mice were placed in a 12cm transmit/receive resonator designed for imaging of wrists. Mice were placed such that the tumors were aligned in the head-foot direction in order to minimize the number of tomographic slices required to include the tumors of both mice. Following acquisition of scout images, a pre-injection baseline scan was required using a spin-echo sequence with parameters: Field of view = $90 \times 53\text{mm}$, reconstruction matrix size = 192×114 , slice thickness = 1mm (10 slices), repetition time = 2070ms, echo times = 11-198ms in 11ms steps (18 echoes), number of averages = 2, scan acquisition time = 7m56s. Following acquisition of the baseline scan, the scanner table was moved (while retaining the isocenter imaging position information) and mice were dosed with either the ferritin conjugate (ferritin + anti-Necl-5 antibody) or a control dose (ferritin alone, no targeting ligand) via direct injection into the tumor without moving the mice. Immediately following injection, mice were returned to the imaging position and time series scans were started. The time series scan used the same parameters as the baseline scan and were carried out in sequential fashion with no pause between scans such that a set of images were acquired every eight minutes for 90 minutes following injection. R_2 maps were computed by taking the baseline and time series scans and performing a three-parameter nonlinear least squares fit of the pixel intensity v. echo time for the expression $M_{xy}(TE) = M_0 e^{-TE/T_2} + DC$ where the fit parameters are M_0 (steady state equilibrium magnetization, i.e. corresponding to $TE = 0$), T_2 , and DC (offset). Mean tumor $1/T_2$ (R_2) values (and standard deviations) were determined by manual segmentation of tumors across all slices in which they appeared for baseline and for each time point in the time series (8 minute intervals).

Statistical Methods

Data were analyzed assuming a Gaussian distribution using PROC GLIMMIX in SAS 9.4® Software for Windows (SAS Institute, Inc., Cary, NC). Significance level was established a priori at $p \leq 0.05$. R_2 , measured in s^{-1} , was modeled with time, measured in minutes, by condition (conjugate vs. control), where observations were nested within mouse. As

a conservative effort, multiple comparisons were calculated using Bonferroni correction. Data were analyzed for both direct and tail vein experiments.

Sample. For the direct injection experiment, 14 mice were randomly assigned to either the conjugate condition (n=6) or the control condition (n=8), and were followed up to 144 minutes. For the tail injection experiment, 12 mice were randomly assigned to either the conjugate condition (n=7) or the control condition (n=5), and were followed up to 80 minutes.

Results. For the direct injection experiment, a significant interaction effect was observed for time by condition ($p < .0001$). Multiple comparisons indicate conjugate scores were higher relative to controls. Specifically, between 56 minutes and 96 minutes, conjugate means were either significantly higher than control means or approached significance ($p=0.030, 0.062, 0.033, 0.017, 0.006$, respectively). In addition, as can be seen in **Figure 4**, between 40 minutes and 144 minutes, the confidence bounds for the two conditions do not overlap. For the tail injection experiment, a significant interaction effect was observed for time by condition ($P < .0001$). Between 48 and 80 minutes, multiple comparisons indicated conjugate scores were either significantly higher than control means or approached significance, with the exception of 64 minutes, which was not significant ($p=0.063, 0.032, 0.13, 0.09, 0.0004$, respectively). In addition, as can be seen in **Figure 2**, the confidence bounds between 8 and 80 minutes do not overlap.

RESULTS AND DISCUSSION

Mice were injected in the rear flank with 1×10^7 PEC SAI cells as described earlier. After two weeks, the tumor size reached 1-1.5 cm in diameter. Mice were divided into two groups, one injected via tail-vein with the Fe(III)-enriched ferritin only and the other with anti-Necl-5/ferritin nanoconjugate. The two groups were scanned using T_2 -weighted, spin-echo imaging at 4 and 24 hours post-injection.

Table 1. Mean R_2 values comparing tumor and surrounding muscle of nanoconjugate and ferritin control injected mice. Significant changes in R_2 MRI contrast values were limited to the nanoconjugate tumor tissue. (Below)

Dose	Baseline	4 hours	24 hours
0.8 mg Conjugate Tumor	13.7 ± 2.0	22.9 ± 5.2	15.2 ± 2.0
Control Tumor	15.9 ± 2.2	13.5 ± 1.7	13.8 ± 1.9
0.8 mg Conjugate Muscle	31.8 ± 4.4	31.0 ± 4.4	29.0 ± 3.7
Control Muscle	33.7 ± 5.1	31.0 ± 4.7	28.7 ± 4.2

Figure 1. R_2 MR images of mice with tumor derived from rat PEC SAI cells suggest utility of anti-Necl-5/ferritin nanoconjugate as a novel contrast agent.

The obtained T_2 data were converted to R_2 ($R = 1/T$). As shown in **Figure 1**, the tumor area indicated by the red arrow in the R_2 overlay map (Upper Right) showed stronger responses than those of the control (Lower Right): $22.9 \pm 5.2 \text{ s}^{-1}$ and $13.5 \pm 1.7 \text{ s}^{-1}$, respectively. Baseline R_2 readings (Upper and Lower Left) were acquired prior to tail vein injections: $13.7 \pm 2.0 \text{ s}^{-1}$ and $15.9 \pm 2.2 \text{ s}^{-1}$, respectively. No dramatic difference was observed in the control animal between baseline and 4 hours post injection (Lower left and right), $15.9 \pm 2.2 \text{ s}^{-1}$ and $13.5 \pm 1.7 \text{ s}^{-1}$, respectively. The nanoconjugate effect diminished at 24 hours to $15.2 \pm 2.0 \text{ s}^{-1}$ but not quite to the baseline level, $13.7 \pm 2.0 \text{ s}^{-1}$ as shown in **Table 1**. This suggests that a strong interaction is present between the conjugate and Necl-5, the tumor-specific antigen, which gradually diminishes over time as the conjugate is cleared out by a continuous hemodynamic flow. Furthermore, uneven R_2 signal distribution is observed at 4 hour in the tumor (Upper right in **Figure 1**), suggesting that within the tumor, Necl-5 is expressed heterogeneously. At this stage, it is not clear that the observed heterogeneity is originated from the accumulation of the conjugate in the tumor stroma or the parenchyma. It was also noteworthy that either the

conjugate or the control R_2 values representing muscle tissues showed little change from the baseline level throughout the 24-hour period. This provided an excellent contrast between the tumor and the surrounding tissue as shown in **Figure 1**.

The interaction between high-molecular-weight iron complexes, such as ferritin and hemosiderin, and water molecules leads to faster dephasing of transverse magnetization, i.e., T_2 and T_2^* reduction, in iron-laden tissues [22, 24]. This is observed as a loss of signal intensity in the tissue. This darkening of the tissue is proportional to the iron concentration [23]. We have attempted gradient-echo (GRE) sequences with T_2^* -based contrast on subcutaneous tumor to illustrate the conjugate containing iron-enriched ferritin (results not shown). However, the resultant T_2^* images showed distortion and signal loss at the tumor, especially for longer echo times (>15 msec) and were not deemed suitable for analysis. This distortion comes from the different magnetic susceptibilities of the tumor tissue (subcutaneous) and air. Where such susceptibility “mismatches” occur, a magnetic field gradient is produced which results in signal loss and geometric distortion. As the GRE sequence did not refocus static field inhomogeneity as does the spin-echo (T_2) sequence, such inhomogeneity resulted in the observed distortion. Thus, the GRE method would be more appropriate for use with tumors located deep inside the body where the local susceptibility changes are less significant (e.g., prostate cancer).

We have confirmed so far that the conjugate administered via tail vein injection gives discernable contrast effect at 4 hour, and the effect can last even at 24 h albeit significantly diminished. The spin-echo sequence was found to be more adequate method than the gradient-echo sequence as the tumor is located near surface. Next, we were interested in the initial kinetic behavior of the conjugate. As described earlier, the conjugate and control (0.8 mg, 300 μ L) were administered via tail vein injection and T_2 reduction was measured using the spin-echo sequence at every 8 minutes for 11 times (80 minutes). As shown below (**Figure 2**), the conjugate R_2 response gradually increases (0-8 min, $\Delta R_2 = 0.92$, $p = 0.014$) as the control (ferritin only) shows initially flat (0-8 min, $\Delta R_2 = 0.05$, $p = 0.999$). This can be interpreted as the conjugate being accumulated in the tumor induced by the conjugate antibody-antigen interaction as designed while untargeted ferritin moves through and eventually out of the tumor. The accumulation may continue beyond the measured time (80 minutes) as it is shown in the earlier experiment (R_2 at 4 hour 22.9 ± 5.2 s $^{-1}$, $\Delta R_2 = +9.2$ s $^{-1}$ from the baseline **Table 1**). It is remarkable to observe the R_2 increase over time (meaning functioning conjugate) despite that the injected conjugate must have gone through multiple hepatic-pass: i.e., average blood-flow rate and total blood volume (mouse, weight 20 g): 1.8 mL/min and ca. 2 mL, respectively.

Figure 2 A plot of R_2 response of the conjugate versus control (ferritin only) over time (80 minutes). The samples were administered via tail vein injections (0.8 mg, 300 μ L).

We further examined the fate and distribution of the conjugate in the tumor and liver tissues by an immunofluorescence assay. For this, we harvested the tissues from euthanized animal at approximately the 6th hour post injection. As shown in **Figure 3**, the control (ferritin only, Panel A) in the tumor tissue did not have any significant signals. On the other hand, the conjugate (Panel B) rendered strong signals at 488 nm (green, Alexa Fluor® 488 Antibody) suggesting that Necl-5 is expressed in high concentration on the tumor cell surface and interacts strongly with the conjugate. It was also noted that the Necl-5 expression was not uniformly distributed in the tumor tissue as suggested earlier in the **Figure 1**. This heterogeneity, more specifically, the parts of tumor tissue with the lack of strong signals may come from the endocytosis of Necl-5 caused by a cell-to-cell contact during proliferation. It has been reported that

the cell-to-cell contact inhibits the cell proliferation and motility processes by down-regulating Necl-5 through endocytosis and inactivating integrin $\alpha_v\beta_3$, respectively [28]. The liver tissue with the control showed a complete absence of 488 nm signals (Panel C) in **Figure 3**. On the contrary, the liver tissue with the conjugate revealed the prevalent 488 nm signals throughout the tissue (Panel D). The conjugate is assumed to be eventually phagocytosed or endocytosed by the reticulo-endothelial system (RES). The initial biodistribution of the conjugate into the cells in the RES depends on the half-life in blood and the size of the conjugate [29]. It is generally believed that nanoparticles with long blood half-lives will have limited distribution into the sinusoidal cells of the liver, i.e., endothelial and Kupffer cells while significant uptake was observed into the RES of other organs, i.e., spleen, lymph nodes and bone marrow. Particulate materials with short half-lives were found in the liver RES cells. Although iron oxide nanoparticles with hydrodynamic diameter less than 40 nm are known to circulate longer than particles with larger diameters, the coating materials used to stabilize the particles may influence both pharmacokinetics and biodistribution of the nanoparticles into the liver [30]. Complexity exists in our nanoconjugate with respect to evaluating biodistribution and pharmacokinetics as the nanoconjugate is comprised of several components: a genetically modified ferritin with microbial origin, iron (III) ions contained in the ferritin and a monoclonal antibody against Necl-5. Nevertheless, the nanoconjugate appeared to have a reasonably long half-life since they were still visible in MRI even at 24 hours albeit examined in an immune-compromised mouse model (**Table 1**). The small size (less than 40 nm in diameter) also seemed to help the nanoconjugate circulating longer than the large-size nanoparticles [21].

Figure 3 Immunofluorescence (Alexa Fluor® 488 Antibody) responses of tumor (Panel A and B) and liver (Panel C and D) tissues acquired from the mice with the tail vein injection of sample (0.8 mg, 300 μ L). The sample is either ferritin only (control Panels A and C) or the conjugate (Panels B and D). Approximately four hours after the MRI T_2 measurement (**Figure 2**) was complete, the mice were sacrificed and the corresponding tissues were harvested. Scale bar is equivalent to 50 μ m.

Direct injection exhibits a very different R_2 profile as shown in **Figure 4**. The increase in R_2 is more significant and rapid as compared to the tail vein responses: First 80 minutes, ΔR_2 , direct (tail vein); for conjugate $+8.32 \text{ s}^{-1}$ ($+1.62 \text{ s}^{-1}$), for control $+3.29 \text{ s}^{-1}$ (-0.55 s^{-1}).

Figure 4 A plot of R_2 response of the conjugate versus control (ferritin only) over time (144 minutes). The samples were directly injected.

The control R_2 values come from the ferritin only in **Figure 4**. As the ferritins are mobile, the observed R_2 values may represent the “unbound” ferritins trapped in various interstitial spaces, thus relatively static, within the tumor parenchyma. This can explain the plateau established by control early in the measurement. In comparison, the conjugates start interacting with Necl-5 expressing tumor cells, which become stationary by the interaction (a typical antibody-antigen dissociation constant $K_d < 10^{-7}$ M) and reach a near-saturation point at 80 minutes as shown in **Figure 2**. Since the R_2 response-time plot is based on a mean value estimation of pixel change of tumor area, it is difficult to evaluate the R_2 distribution pattern within the tumor. The distribution patterns of the conjugate and control via direct injection are illustrated in **Figure 5**. Transverse plane views obtained from a 90° rotation of the cross-section (a dotted-line in **Figure 5a**) represent overlays of R_2 response images over time as shown in **Figure 5b**. For the control, magnitude of R_2 change over time (i.e., baseline, 24 minutes and 90 minutes) is minimal. In contrast, the color change at the center of the tumor is apparent at 24 minutes, and at 90 minutes red spots ($R_2 \approx 40\text{-}50\text{ s}^{-1}$) are formed as nodules spread out the entire tumor. Since the conjugate can only interact with Necl-5 that is expressed only on rapidly growing tumor cells, it is believed that the nodule-like spots are the tumor stromal cells.

Figure 5 a) A coronal posterior view of a mouse directly injected with the samples (0.8 mg, 300 μ L) in MRI. A dotted line is a cross-section that gives a transverse plane with a 90° rotation. The arrow indicates the location of tumor. **b)** The transverse plane images are shown for mice, directly injected with ferritin only (control) and the conjugate at baseline (pre-injection), 24 minutes and 90 minutes post-injection.

CONCLUSIONS

The nanoconjugate interacts strongly and selectively in situ with Necl-5 overexpressing tumor cells. Direct injection of the nanoconjugate into the body of the tumor caused a more significant in situ R_2 increase in MRI than the tail vein injection. Varying degrees of R_2 increase within the tumor mass is likely to represent different distribution patterns of the conjugate, reflective of tumor heterogeneity.

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Conflict of Interest The authors declare that they have no conflict of interest.

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