Toxicity and Pharmacokinetics of uPAR-targeted Human ATF-conjugated Iron Oxide Nanoparticles Following Systemic Delivery in rhesus Monkey

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ABSTRACT

To translate uPAR-targeted human ATF-IONPs into clinical applications, we evaluated MRI changes from Pre- to Post-17 days, serum iron concentrations at from Post- 5 mins to 12wks, routine blood examination and serum chemistry analyses from pre- to 12wks after administration, and stained with hematoxylin-eosin (HE) and Perls' Prussian blue of the liver at post-24 hours and 3 months in rhesus monkeys respectively injected with human ATF-IONPs with and without PEG coating. It is proved that systemic delivery of 5 mg/Kg of iron equivalent concentration of uPAR-targeted human ATF-INOPs is safe. And there were transient alterations in all of the observed variables, and were self-healing in next three months. Therefore, results of our study in monkeys support the potential of future development of uPAR-targeted IONPs as receptor-targeted MRI contrasts as well as theranostic agents for the detection and treatment of human cancers.

Keywords: Nanoparticle, uPAR, MRI, Pharmacokinetic, Monkey

1 INTRODUTION

Increasing evidence supports the potential of a novel cell surface receptor-targeted MRI nanoprobe produced by conjugation of recombinant amino-terminal fragment (ATF) peptide of urokinase plasminogen activator (uPA) to magnetic iron oxide nanoparticles (IONP) in targeted cancer imaging and therapy[1]. To translate this receptor-targeted MR imaging probe into clinical applications for cancer detection and image-guided cancer therapy, preclinical studies in large animal models are necessary to determine systemic toxicity, biodistributions, dose, imaging capability, and pharmacokinetics (PK). In this study, we examined toxicity and PK of uPAR-targeted human ATF-conjugated IONPs in rhesus monkeys. We further evaluated the feasibility of non-invasive MRI monitoring IONPs accumulation in the major organs following systemic delivery.

To our knowledge, this is the first study to examine dynamic changes of MRI signals following intravenous administration of receptor targeted IONPs in monkeys. Our results reported here provide important preclinical information concerning MR imaging capability, PK and systemic toxicity of uPAR targeted IONP with or without a Polyethylene glycol (PEG) modification in rhesus monkeys.

2 METHOD

2.1 Preparation of uPAR-Targeted ATF-IONPs

10 nm core size magnetic iron oxide nanoparticles (IONP) were obtained from Ocean Nanotech, LLC, San Diego, CA.T. The IONP coated with amphiphilic polymers, without or with an additional layer of PEG were conjugated with human ATF peptide produced from a bacterial expression system via a covalent bond between carboxyl groups of the amphiphilic polymers and amino side groups of the peptides at a ratio of one IONP :15 ATF peptides. The final ATF-IONP or ATF-PEG-IONP conjugates were purified using nanosep 100k column. The targeted-ATF-IONP or ATF-IONPs were tested and showed that they are free of endotoxin contaimination.

2.2 Animal Preparation

Two health young rhesus monkeys (4 ± 1 years old; 3.8 ± 0.1kg; 2 males) were initially obtained from the Sichuan Primed Bio-tech Co, Ltd; Chengdu, China. Animals were housed individually in the West China Experimental Animal Center at the Sichuan university. After intravenous anesthesia using 3 to 5 ml of 3% pentobarbital sodium administraed at 2 ml/min, Monkey #1 received intravenous administration of the 5 mg/Kg of Fe equivalent dose of ATF-IONP and Monkey #2 received 5 mg/Kg of Fe equivalent dose of ATF-PEG-IONP.

2.3 MRI Methods

MRI scan was performed on a clinically available 3.0 T Siemens Trio system (Siemens, Erlangen, Germany) with contiguous electrocardiography and breath-hold monitoration of the rhesus monkeys. MRI scan using a surface coil were performed on monkeys before Pre and Post-10, 30, 60 min, 1, 7

and 17 days after intraveneous administration of the IONPs. T2-weighted imaging [TR/TE=3200/65 ms, slices: 15, section thickness=1 mm, Averages=3,] and T1WI [TR/TE=500/12.0ms, slices:25, slice thickness=1mm, Averages=6] were scanned. The regions of interest (ROI) method were used to assess the IONPs induced changes in MRI signal level, contrast, and T2 values in the liver and spleen. The signals of back muscles, which showed little change before and after the injection of the ATF-IONPs, were used as a baseline for comparison of the signals in ROIs.

2.4 Serum Iron Concentrations and Toxicity

For quantification of IONPs in the blood following systemic delivery, blood samples were collected at different time points, including 5 min, 1, 3, 6, 12, 24 and 48 hours, 4 and 10 days, and 2, 4 and 12 wks after injections. The blood samples were subjected to a digestion process in acid. The resulting solutions were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine the amount of Fe in the blood, which represents the amount of IONPs. To evaluate systemic toxicity of the IONPs, we collected blood samples at different times follow the IONP administration. Serum levels of alanine transaminase (ALT), alkaline phosphatase (ALP), and direct bilirubin (BIL-D) for the liver function, creatinine (CREA) for the kidney function, and creatine kinase (CK) for the cardiac function were determined. The routine blood examination (mainly the WBC, RBC and PLT) were conducted Pre-7 days and Post- 2 days, 2, 4, 8 and 12 wks.

2.5 Histological Analysis

Biopsy samples of the liver were obtained from rhesus monkeys at 24h and 3m after IONP injection using a core needle biopsy. The samples were placed in 10% formalin and stained with hematoxylin-eosin (HE) and Perls' Prussian blue for optical microscopy. Microscopy was used to determine acute inflammatory changes or necrotic areas associated with the administration of the IONPs, and for highlighting the iron deposits in Kupffer cells of the Sinusoids of the liver tissue sections.

3 RESULTS

3.1 Toxicity and pharmacokinetics of IONPs following systemic delivery

With intravenous administration, uPAR targeted IONPs are demonstrated to be safe for systemic delivery. Serum iron level from ICP-MS showed that both human ATF-IONP and human ATF-PEG-IONP probes have fast washout time after injection, and almost little residue in the circulation (Fig 1A). Non-compartmental analysis of the iron concentrations of plasma samples obtained at different time following the IONP administration were used to calculate PK (Fig 1B). Although a similar T1/2 found in the monkeys that received two different IONPs, it seemed that the area under the curve(AUC), an indicator of IONP bioavailability in plasma, in ATF-PEG-IONP injected monkey was much larger, compared with ATF-IONP injected monkey.

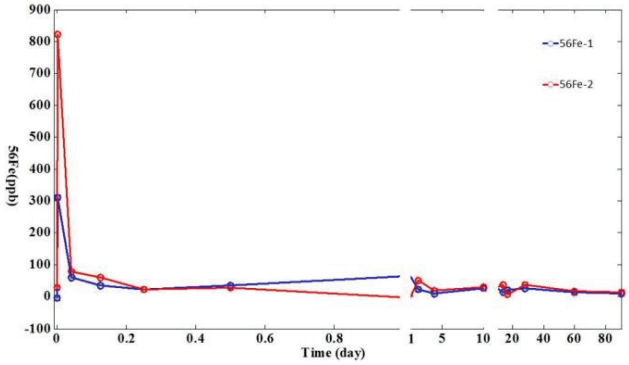

Figure 1A Vriation of serum iron with the injection of IONPs Bule is Monkey #1, red is Monkey #2

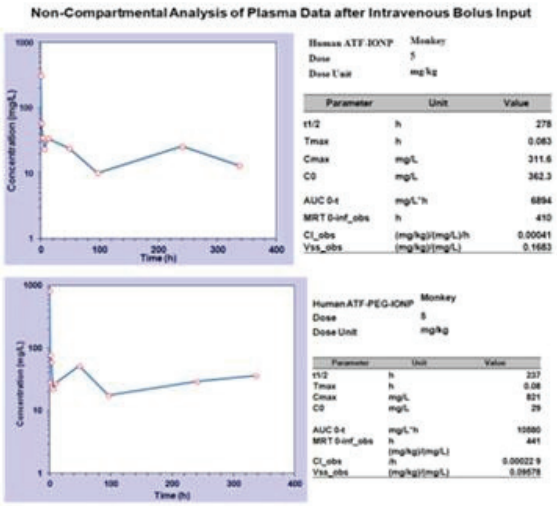

Figure 1B. Determination of PK of uPAR-targeted IONPs in rhesus monkeys. Upper: A monkey received 5 mg/Kg of ATF-IONP. Lower: 5 mg/Kg of ATF-IONP

The levels of ALP2L, ALTL, ASTL values were immediately increased after the administration, and recovered to the pre-contrast after 2~3 months (Figure 2A~C). Indicators of renal function, such as CREA and BIL-D, had the same up and down trends between two rhesus monkeys. After the nanoparticles injection, the values decrased and slight recovery as time goes by (Figure2D,E). CKL values were ascending and descending obviously in Monkey #1, and relatively mild alteration with the similar trend in Monkey #2 (Figure 2F). Compared with the normal reference range provided from over 100 normal rhesus monkeys in Sichuan Primed Bio-tech Co. Ltd; Chengdu, China, we concluded that most of the variables had recovered into normal range within 2 weeks after the IONP delivery.

3.2 Monitoring the Delivery of the IONPs by noninvasive MRI

Magnetic nanoparticles typically lead to reduced MRI signals in T2-weighted imaging in the area where nanoparticles accumulate (Figure 3A,B). We found that the signal intensity of T2WI decreased 28%, 29% in the liver of Monkey #1 and #2 respectively, following systemic delivery of human ATF-IONPs and Human ATF-PEG-IONPs for 10min**.** The decrease in T2 signal intensity retained in the liver till 17 days after the injection (Fig 3). The liver of the Monkey #2 that received human ATF-PEG-IONPs appeared having a higher T2 signal intensity than IONPs without PEG coating in the liver, indicating the effective reduction of nonspecific uptake of the a ATF-PEG-IONP in the liver. T2-signal intensity in the spleen in both monkeys were decreased after administration and stayed at a relative lower lever until Post- 17day. Meanwhile, T1 signal intensity of liver and spleen also diminished immediately after IONP administration, and remained at a low level 17day later (Fig 3). Prussian blue staining analysis of Fe presence of IONP in the tissue sections confirmed that ATF-IONPs reserved in the liver at more than 3m after injection (Fig 4).

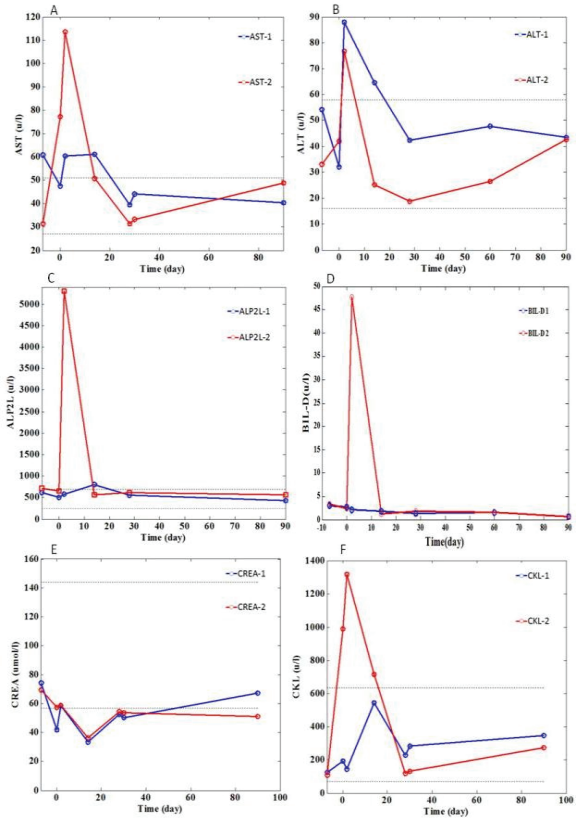

Figure 2 Plots of time dependent changes of IONP contents in rhesus monkeys, suggesting the PK effects of ATF-IONPs as indicated by serological examination. The dash lines show the normal reference range provided by Sichuan Primed Bio-tech Co, Ltd; Chengdu, China.

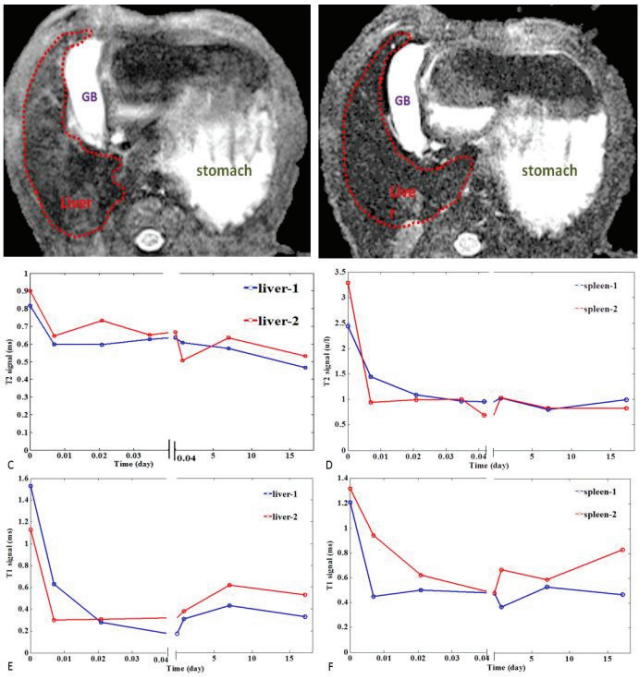

Figure 3 the MRI signal alteration of liver and spleen, A and B are the MR images at Pre- and Post- with ATF-IONP injection, both of them are phased from up to down. C and D are the variation of T2 signal; E and F are the variation of T1 signal.

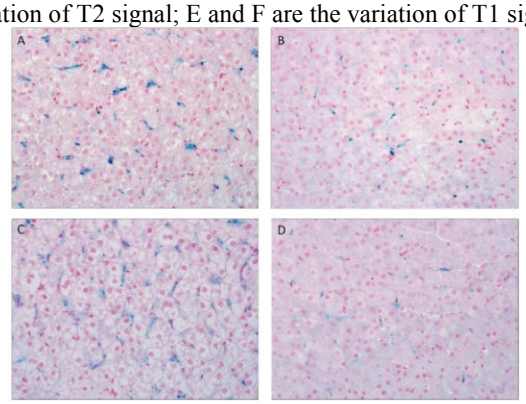

Fig 4. Detection of IONPs in the liver of the rhesus monkeys by Perls Prussian blue staining. The liver of the Monkey #2 had weaker blue staining conpared with Monkey #1 (A and C). And The levels of IONPs in the liver of monkeys were significantly descreased at Post- 3m. And no significant difference between B and D.

4 DISCUSSION

Recently, biocompatible and functionalized nanoparticles have been shown to target tumors and produce optical, magnetic and/or radioactive signals for enhancing sensitivity and specificity of non-invasive tumor imaging. Previous studies have shown the feasibility of producing such imaging probes for in vivo MRI of cancers [2].

Results of this study showed that systemic delivery of 5 mg/Kg of iron equivalent concentration of uPAR-targeted human ATF-INOPs is safe in the rhesus monkey. The results of

toxicity and PK study in two normal rhesus monkeys further demonstrated that after intravenous administration, the T1 and T2 relaxation time of the liver and spleen were shorten, resulting in a signal intensity decrease, which was consistent with our preliminary work that the magnetic IO nanocrystal used in this study has strong effects on shortening both T1 and T2 relaxation times [3]. Histology anlysis results showed that nonspecific accumulated the probes still existed in the liver three months after IONP delivery. And the early research indicated that the accumulated probes were only detected in the liver and spleen and not in other normal organs [3]. It is well known that nanoparticles can be taken up by the reticuloendothelial system in the liver and spleen, and then are subsequently metabolism or utilized for iron storage [4,5]. We also found that the intravenous injection of probes led to nonspecific uptake by the Kupffer cells in the liver (Fig 4) and macrophages in the spleen [3]. In the normal monkeys, this nonspecific accumulation of IONPS in the liver and spleen provided us an opportunity to determine the MRI signal of ATF-IONP or ATF-PEG-IONP follow in vivo accumulation in target organs and feasibility of the MRI scan parameters using 3T MRI scanner developed in mice for MRI of large animals or future MRI in humans.

Meanwhile, extensive studies have shown that decline in liver function occurs with age and with hepatic abnormity and is associated with reduced capacity for drug metabolism, particularly for drugs undergoing oxidative biotransformation [6]. Navarro et al. found that after use arsenic, as AST and ALT are reliable determinants of liver parenchymal injury, increment of the activities of AST and ALT in serum may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream [7], which might be the inflammatory response [8]. Kumari et al. reported that in nanoparticle treated animals, ASAT, ALAT and LDH levels increased in serum with simultaneous decreases in kidney indicating tissue necrosis and possible leakage of these target enzymes into the blood stream [9]. Our result show that the transient alteration of variables indicated the function of liver, kidney, and heart, such as ALT, ALP, BIL-D was self-healing during a short time period, and recoveryed back to the normal level. The same trend was observed by HE staining which means there may be a mild and acute response of the liver caused by accumulation of uPAR-targeted human ATF-INOPs probes in the liver and spleen. However, it is reversible and this effect has to be further cofirmed by increasing the number of monkeys in future studies.

5 CONCLUSSION

This study demonstrated that 5 mg/Kg of iron equivalent concentration of uPAR-targeted human ATF-INOPs are safe for systemic administration in the rhesus monkey. The transient alteration of normal organ functions are observed, and was reversible. Strong MRI signals were nonspecifically accumulated in Kupffer cells. Therefore, it is feasible to use uPAR-targeted human ATF-IONPs or ATF-PEG-IONP for targeted tumor imaging or to deliver therapeutic agents into tumor cells for monitoring the response to therapy by non-invasive MRI. Therefore, results of our study in monkeys support the potential of future development of uPAR-targeted IONPs as receptor-targeted MRI contrasts as well as theranostic agents for the detection and treatment of human cancers.

6 ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (81130027), the National "Twelfth Five-Year" Plan for Science & Technology Support (2012BAI23B08) and the National Basic Research Program of China (973 Program, 2011CB935800).

REFERENCES

[1] Yang L, Peng XH, Wang YA, Wang X, Cao Z, Ni C, Karna P, Zhang X, Wood WC, Gao X, Nie S and Mao H, "Receptor-Targeted Nanoparticles for In VivoImaging of Breast Cancer," Clin Cancer Res, 15, 4722-4732, 2009.

[2] Nasongkla N, Bey E, Ren J, Ai H, Khemtong C, Guthi JS, Chin SF, Sherry AD, Boothman DA and Gao J, "Multifunctional polymeric micelles as cancer-targeted, MRI ultrasensitive drug delivery systems." Nano Lett, 6, 2427–2430, 2006.

[3] Lee GY, Qian WP, Wang L, Wang YA, Staley CA, Satpathy M, Nie S, Mao H and Yang L, "Theranostic Nanoparticles with Controlled Release of Gemcitabine for Targeted Therapy and MRI of Pancreatic Cancer," ACS Nano, 7, 2078-2089, 2013.

[4] Bulte JW and Kraitchman DL, "Iron oxide MR contrast agents for molecular and cellular imaging," NMR Biomed, 17, 484–499, 2004.

[5] Thorek DL, Chen AK, Czupryna J and Tsourkas A, "Superparamagnetic iron oxide nanoparticle probes for molecular imaging," Ann Biomed Eng, 34, 23–38, 2006.

[6] Cawello W, Fichtner A, Boekens H and Braun M, "Influence of hepatic impairment on the pharmacokinetics of the dopamine agonist rotigotine," Eur J Drug Metab Pharmacokinet, doi 10.1007/s13318-013-0153-x, 2013.

[7] Navarro, C.M., Montilla, P.M., Martin, A., Jimenez, J., Utrilla, P.M., 1993. "Free radicals scavenger and antihepatotoxic activity of Rosmarinus," Planta. Med, 59, 312–314, 1993.

[8] Kim TY and Kimk DJ, "Acute-on-chronic liver failure," Clin Mol Hepatol, 19, 349-359, 2013.

[9] Kumari M, Rajak S, Singh SP, Kumari SI, Kumar SI, Murty US, Mahboob M, Grover P and Rahman MF, "Repeated Oral Dose Toxicity of Iron Oxide Nanoparticles: Biochemical and Histopathological Alterations in Different Tissues of Rats," J Nanosci Nanotechnol, 12, 2149-2159, 2012.