Supporting information for

Arsenic Trioxide Nanoparticle Prodrug (ATONP) Potentiates Therapeutic Effect on an Aggressive Hepatocellular Carcinoma Model via Enhancement of Intratumoral Arsenic Accumulation and Disturbance of Tumor Microenvironment

Xin Fu[a], Qing-rong Liang[d], Rong-guang Luo[b], Yan-shu Li[c], Xiao-ping Xiao[d], Lu-lu Yu[d], Wen-zhe Shan[d], Guang-qin Fan[a], Qun Tang[a,d]*

[a]Jiangxi Provincial Key Laboratory of Preventive Medicine, School of Public Health, Nanchang University, Nanchang, 330038，P. R. China
[b]Department of Medical Imaging and Interventional Radiology, the First Affiliated Hospital of Nanchang University, Nanchang, 330006, P. R. China
[c]Jiangxi Supervision and Inspection Center for Medical Devices, Nanchang, 330029, P. R. China
[d]Institute for Advanced Study, Nanchang University, Nanchang, 330038, P. R. China

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Deduction of chemical formulation for inorganic synthesis of ATONP

We propose the synthetic process is a pH-determined reversible reaction, taken gadolinium as an example, as long as gadolinium chloride ($GdCl_3$) was fully mixed with equivalent amount of sodium arsenite ($NaAsO_2$) (e.g. [$GdCl_3$] = [$NaAsO_2$] = 1M), the reaction was initiated, and the solution tends to become more acidic because of production of hydrogen chloride (HCl):

$$GdCl_3 + NaAsO_2 + H_2O \rightleftarrows GdAsO_3(\text{colloid}) + NaCl + \mathbf{2HCl} \text{ (equation 1)}$$

Simultaneously, the as-produced hydrogen chloride (HCl) could react with one of the reactant sodium arsenite ($NaAsO_2$), and forming stable arsenic acid (H_3AsO_3), thus, as the forward reaction goes, more hydrogen chloride was produced and sodium arsenite was exhausted in the way as bellows:

$$HCl + NaAsO_2 + H_2O = H_3AsO_3 + NaCl \text{ (equation 2)}$$

The chemical equilibrium between AsO_2^-, H^+, and H_3AsO_3 was determined by dissociation constant of arsenic acid (H_3AsO_3):

$$pKa(H_3AsO_3, 9.22) = [AsO_2^-]*[H^+]/[H_3AsO_3] \text{ (equation 3)}$$

So, the pH value in the aqueous solution can be approximately functioned with reaction percentage (RP), defined as the reaction mass divide the total mass of reactant:

$$(1-3*RP+10\text{^}(-pH))/(2*RP-10\text{^}(-pH)) = 6*10\text{^}(-10);\ RP \leq 1/3 \text{ (equation 4)}$$

At the point that RP arrived at one third (RP=1/3), two third gadolinium chloride ($GdCl_3$) was used, but almost all the sodium arsenite ($NaAsO_2$) were exhausted, because two third sodium arsenite ($NaAsO_2$) was exhausted according to equation 1, and two third was proposed to formation of arsenic acid (H_3AsO_3) (equation 2). The reaction is expected to be terminated as RP reach 1/3, as analyzed above, according to equation 4, pH can be plotted against with RP, as figure S1a shown, as long as RP exceed 1/3, even a tiny forward progress causes huge pH to drop down. Moreover, if this formulation is correct the theoretical terminated pH is determined by initial reactant concentration, and higher concentration produces more acidic colloidal solution, according to the equations, we calculated the theoretical pH value under different initial reaction concentration, and they are very close to the experimental data (figure S1b).

Direct elemental distribution test further confirmed that approximate one third of gadolinium and arsenic distributed into the $GdAsO_3$, and two third (2/3) was left in the supernant, as shown in figure S1c. Therefore, it is reasonable that if alkalization of the residual supernant containing two third of gadolinium and arsenic the reaction can be continuously recycled (figure S1c), and during the successive circular reaction, initial reactant's concentration gradually reduces, as we previously demonstrate this reduction leads equilibrium pH higher and higher, as proved in figure S1d. This recyclable characteristic makes the synthetic process economical and environment-friendly. TEM images illustrated that even after three recycles, $GdAsO_3$ keep stable morphology by statistical calculation (not shown).

This inorganic reaction is intentionally terminated by a certain time (30 min), and elongation reaction time only produced longer nanorod with its diameter remained, but the element distribution did not change at all.

Furthermore, re-treatment the separated supernant or colloid without alkalization does not initiate any reaction but restarting the mixture of supernant and colloid give longer nanowire without ratio distribution variance.

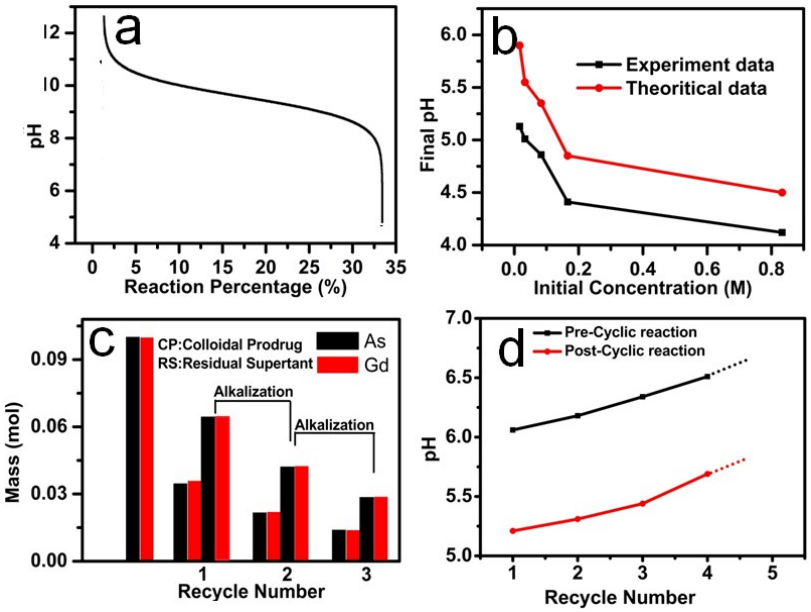


Figure S1. (a) Theoretical curve of equation 4, reaction percentage in accordance with the abscissa, the ordinate pH values for reaction curve, M_0=[$GdCl_3$] = [$NaAsO_2$] = 1mol/L. As the reaction percentage is close to one third (1/3), the solution pH value drops down sharply. (b) Comparison of experiment data (initial concentration *vs* measured final pH) and theoretical data calculated according to equation 1-4. (c) Elemental distribution during ATONP formation and double alkalization. During the synthetic process one third (1/3) Gd and As was internalized into ATONP, while the residual supernant kept the rest two third (2/3), alkalization was taken by adding equivalent sodium hydroxide to arsenic acid (H_3AsO_3) after separation by high-speed centrifugation, re-start the chemical reaction (equation 1, 2).

Physicochemical analysis of ATONP

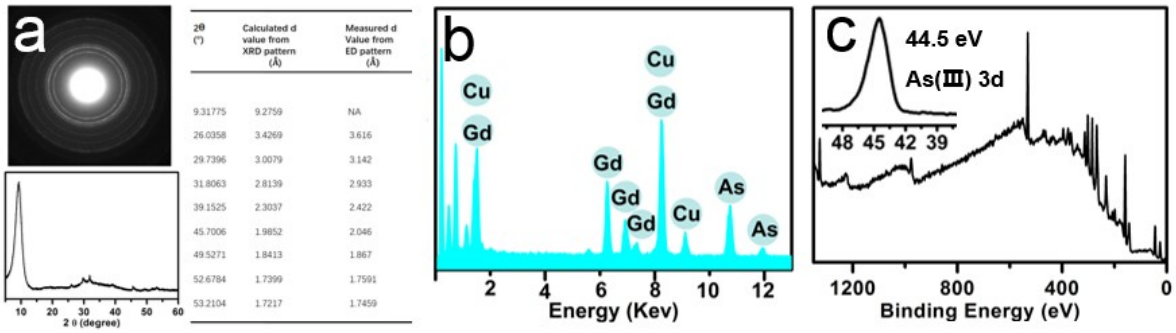
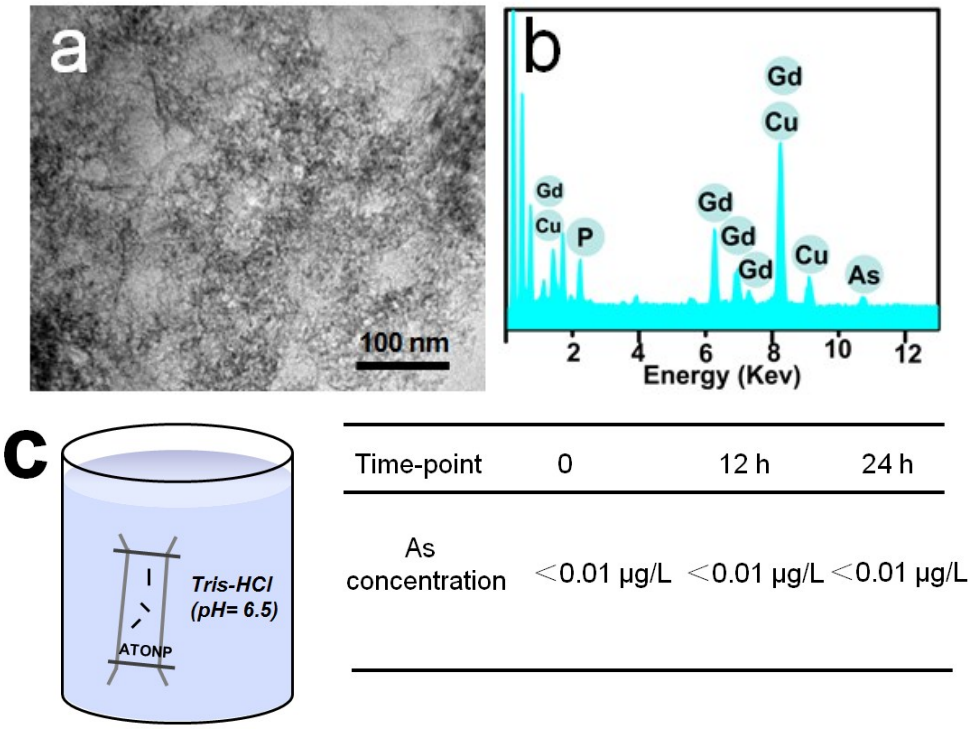


Figure S2. (a) Electron diffraction pattern recorded on GdAsO3 showed consistent with X-ray diffraction pattern of GdAsO3. (b) Gd and As are identified by EDX equipped with TEM, the elemental ratio is close to 1:1. (c) X-ray photoelectron spectroscopy recorded on the lyophilized GdAsO3, the peak located at 44.5 eV is assigned to the As(Ⅲ)-O binding energy.

Note: There is no figure-printed XRD pattern in the JCPDS datadabase for $GdAsO_3$, and we have not got its single crystal for phase index, so the exact phase and structure of $GdAsO_3$ cannot be identified, but electron diffraction pattern are highly matched with the XRD pattern, which confirms

the phase purity of gadolinium arsenite ($GdAsO_3$).

3. Product after dialysis and Pi-free dialysis in weak acid



Time-point	0	12 h	24 h
As concentration	<0.01 μg/L	<0.01 μg/L	<0.01 μg/L

Figure S3. (a) TEM image of the ion-exchanged product within the dialysis tube. (b) EDX showed the most arsenic has released with gadolinium phosphate left. (C) Scheme of ATONP (1mg) sealed in the dialysis tube dialyzed against in the simulated TME (tris-HCl, pH 6.5) solution (50 ml). At three time-points (before dialysis, 12 hours and 24 hours after dialysis) arsenic concentration outside the dialysis tube are all undetectable by ICP-AES.

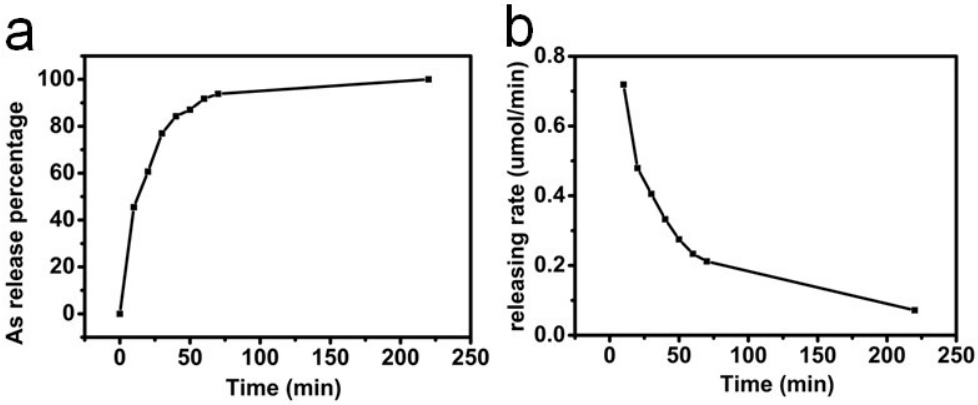


Figure S4. (a) Arsenic releasing percentage curve and releasing rate as ATONP was directly mixed with human serum at 4 degree.

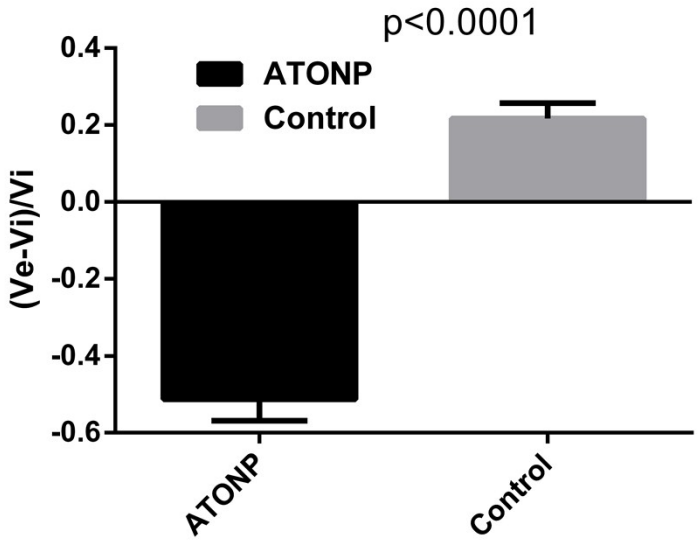


Figure S5. Tumor volume change in ATONP group vs saline group. Ve refers to the volume of end point, and Vi means initial volume.

4. Acute toxicity of ATONP

group	Injected dose		NO. Dead/ NO. Injected		Mortality (p)	Survival rate (q)
	(mg As/kg)	logarithm	Male	Female		
1	5.75	0.76	0/4	0/4	0	1
2	6.92	0.84	2/4	1/4	0.375	0.625
3	8.32	0.92	3/4	3/4	0.75	0.25
4	10	1	4/4	3/4	0.875	0.125
5	12.02	1.08	4/4	4/4	1	0

Table S1. Acute toxicity of $GdAsO_3$ in Balb/c mice. $GdAsO_3$' LD_{50} = 7.59 mg As/kg (95% confidence limit of 8.24-6.98 mg As/kg).

5. Reversible sub-chronic toxicity of ATONP

	Treated groups		
	Control (PBS)	Group A	Group B
Bone Marrow Function			
WBC(*10^9/L)	1.2±0.25	2.85±0.38	1.3±0.46
RBC (M/μL)	7.1±0.36	7.5±0.56	7.2±0.28
HGB (g/dL)	12.3±3.2	13.56±5.1	12.9±2.8
PLT (*10^9/L)	860±91	752±81	873±108
HCT (%)	30.5±8.5	32.9±4.2	29.8±9.7
Liver function			
Total Protein (g/dL)	5.5±0.5	5.3±1.2	5.4 ±0.3
Albumin (g/dL)	2.3±0.4	2.5±0.2	2.4±0.5
Cholesterol (mg/dL)	88.5±10.2	90.2 ±8.2	92.8±9.4
ALT (U/L)	30.8±7.4	40.1±5.4*	32.5±4.9
ALP (U/L)	41.5±13.8	55±10.9*	40.7±9.8
Total Bili (mg/dL)	0.24±0.1	0.27±0.1	0.25±0.08
Renal function			
Urea Nitrogen (mg/dL)	22.7 ±0.8	29.8±2.7	19.7±2.5
Creatine (mg/dL)	0.08±0.02	0.04±0.02*	0.09±0.03
Phosphorous (mg/dL)	7.9±2.1	6.4±1.2	8.7±1.9
Calcium (mg/dL)	10.2±0.9	6.9±0.7	9.7±0.7

WBC: White blood cell; RBC: red blood cell; HGB:Hemoglobin; PLT:Platelet; HCT: hematocrit; ALT: Alanine aminotransferase; ALP :alkaline phosphatase. (n=6 mice).

Table S2: Peripheral blood count and serum chemistries

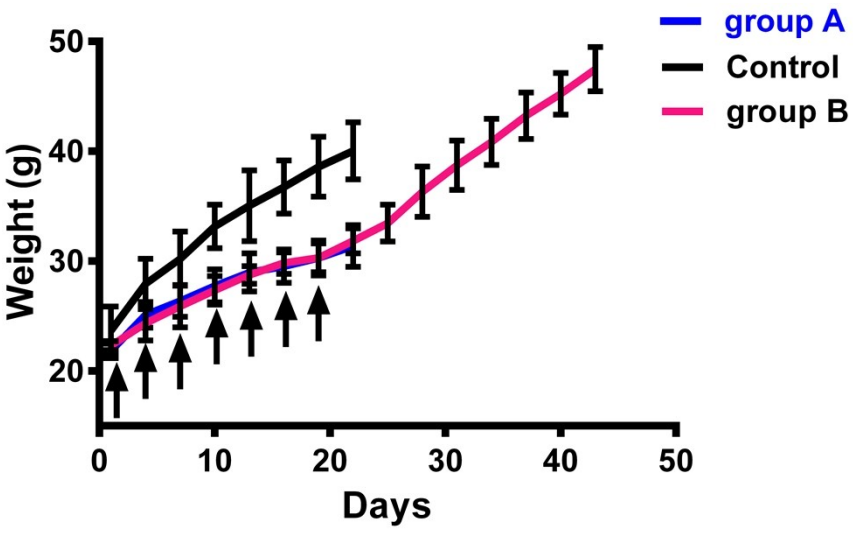


Figure S6. The weight change of administered mice in the three groups. The mice in group A and B showed slower growth rate than control group during the administration period, which hints it might suffer from toxicity, but the weight increased slightly faster after withdraw of ATONP.

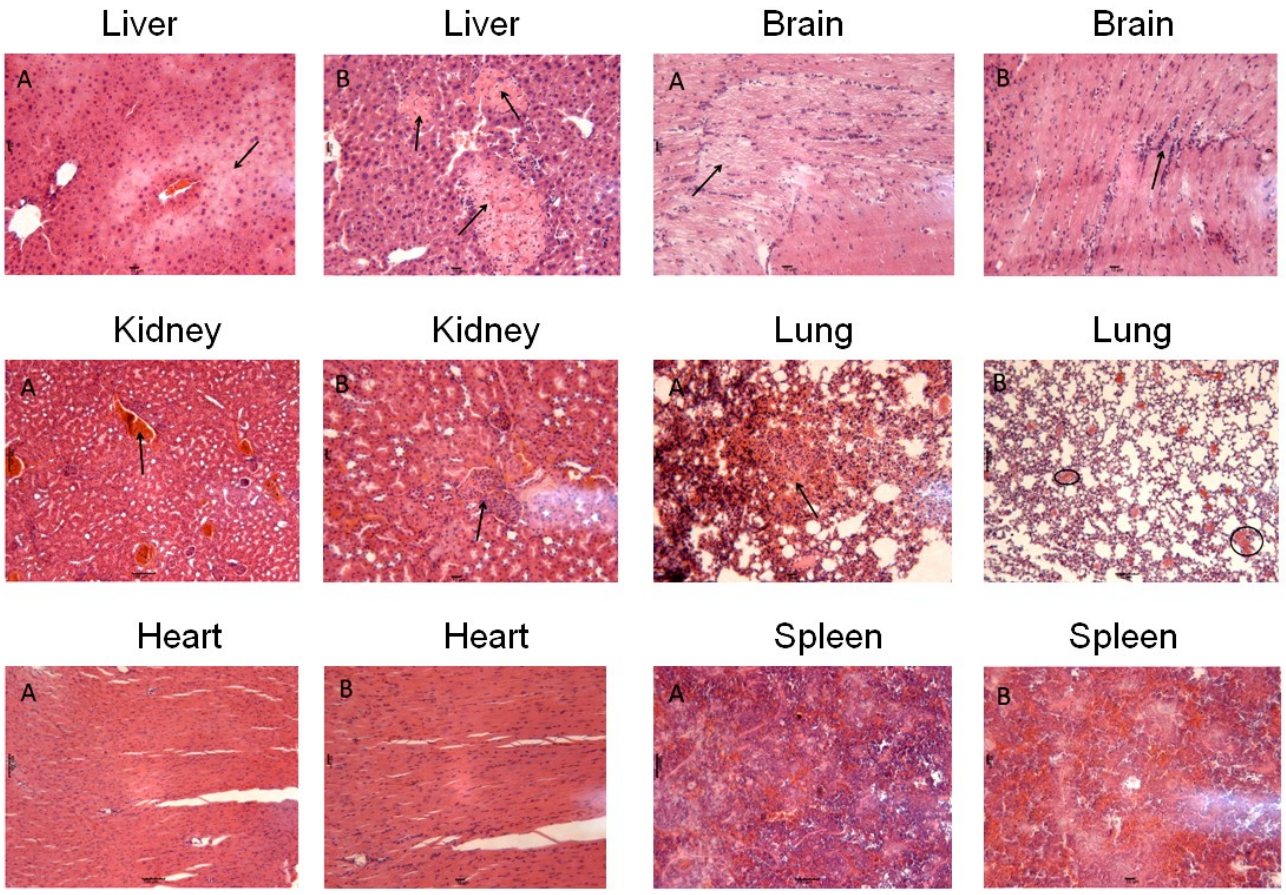


Figure S7. Representative fields of H&E stained tissues (liver, brain, kidney, lung, heart, spleen) of mice in group A.

Liver: Hepatocytes are partially swollen and fatty degeneration are occasionally visible, and the sinusoid partially disappeared (A 100*magnification)； Hepatic lobule is partially arranged with cell degeneration, coagulation necrosis, and inflammatory cell infiltration (B 400*magnification)
Brain: nerve fiber is partially sparse, edema degeneration is visible (A 100*magnification)，Glial cell is occasionally infiltrated and aggregated (B 400*magnification)
Kidney: Moderate congestion (A 100*magnification)，Renal capsule shrink significantly, glomerular cells increased (B 400*magnification)。
Lung: Pulmonary hemorrhage (A 400*magnification)，Also visible pulmonary alveolar homogeneous pink staining of protein-like material exudation, but also see the individual small arterial thrombosis (B 100*magnification).
Heart: normal (A 100*magnification; B 400*magnification).
Spleen: Lymph nodules have reduced the trend, showing increased proliferation of multinucleated macrophages, sinusoidal expansion, lymphopenia, tissue cell proliferation, spleen hematopoietic dysfunction (A 100*magnification; B 400*magnification).

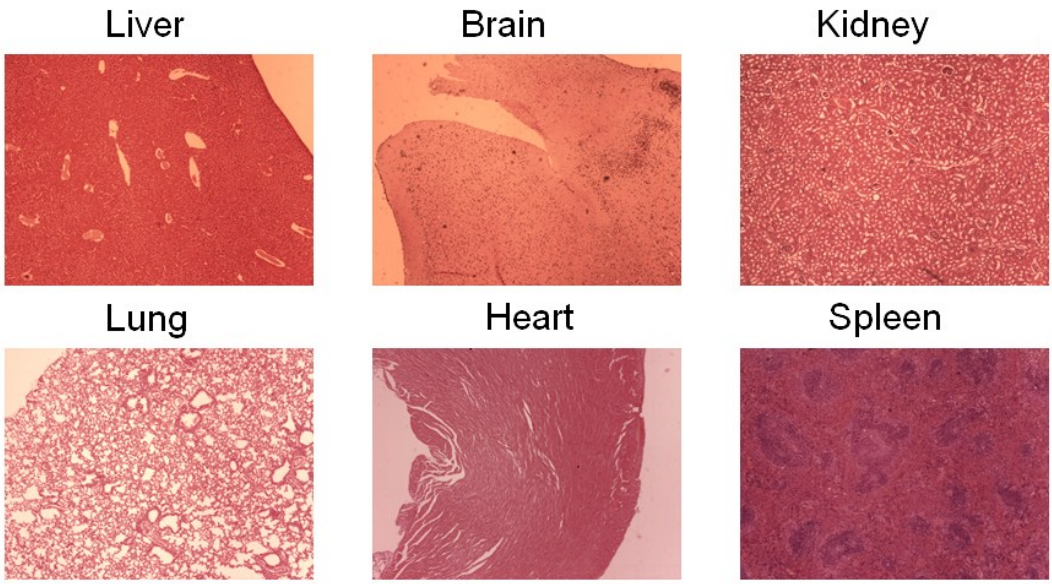


Figure S8. Representative fields of H&E stained tissues (liver, brain, kidney, lung, heart, spleen) of mice in group B and group C. No abnormal histology was observed in both groups.

a

Samples NO.	ATONP solution (1mg/ml) (ml)	physiological saline (ml)	2% erythrocyte suspension (ml)	water (ml)	hemolysis
1	0.5	2	2.5	0	N
2	0.3	2	2.5	0	N
3	0.1	2	2.5	0	N
4	0	2	2.5	0	N
5	0	0	2.5	2.5	Y

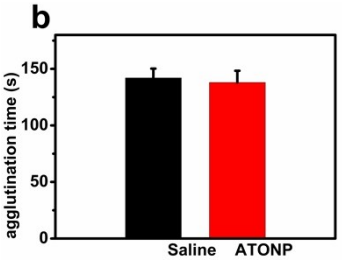


Figure S9. (a) Hemolysis test at various volumes of 1 mg As/ml with negative (sample 4) and positive (sample 5) control experiments. (b) Hemagglutination test on the control solution (saline) versus 0.4 mg As/kg injection dose.

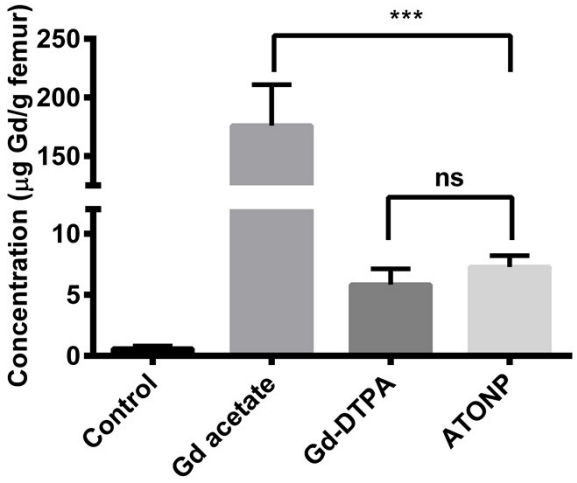


Figure S10. Gadolinium accumulation in the femur after administration of equivalent dosage of Gd acetate, Gd-DTPA and ATONP, and only saline was administrated as control.