Supporting Information

A Glutathione-Specific and Intracellularly Labile Polymeric Nanocarrier for Efficient and Safe Cancer Gene Delivery

Guowei Wang†, Dingcheng Zhu†, Zhuxian Zhou*, Ying Piao, Jianbin Tang, and Youqing Shen*

Center for Bionanoengineering and Key Laboratory of Biomass Chemical Engineering of Ministry of Education, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China

* Corresponding author, e-mail: zhouzx@zju.edu.cn or shenyq@zju.edu.cn
† These authors contributed equally to this work
Supplementary Experiments

1. Materials. Glutathione (GSH), cysteine (Cys), homocysteine (Hey), sodium hydrosulfide (NaSH), N-bromosuccinimide, p-cresol, 1-fluoro-2,4-dinitrobenzene, 4-bromomethylbipheny, 1, 2-(N, N-diethylamino)ethyl acrylate (DEEA), linear and branched polyethylenimine (PEI, 25 kDa) were purchased from Sigma-Aldrich (Shanghai, China). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS) and N-acetylcysteine (NAC) were purchased from Tokyo Chemical Industry Co., Ltd (Japan). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) was purchased from Laysan Bio. Inc (USA). L-buthionine sulfoximine was purchased from Acros Organics (Belgium). pGL4.13 luciferase plasmid (pLuci) and luciferase assay system were purchased from Promega (Madison, WI). Enhance green fluorescent protein plasmid (pEGFP) and tumor necrosis factor-related apoptosis inducing ligand receptor plasmids (pTRAIL) were provided kindly by Zhejiang University School of Medicine and Shanghai Institute of Materia Medica, Chinese Academy of Sciences. One Step TUNEL Apoptosis Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). FITC Annexin-V Apoptosis Detection Kit was purchased from BD Pharmingen (USA). LabelIT® Nucleic Acid Labeling Kit of Cy5™ was purchased from Mirus Bio (Madison, WI, USA). Lipofectamine 2000 (Lipo2000) was purchased from Invitrogen, USA. Poly(2-N, N-diethyl)aminoethyl acrylate) (PDEAEA) and PDEAEA functionalized with primary amines (PDEAEA-NH2) was synthesized as our previous reports.30

2. Cell culture and animals. Adenocarcinomic human alveolar basal epithelial cells (A549), human cervical carcinoma cells (HeLa), human hepatoblastoma-derived cells (HepG2) and normal fibroblast cells (NIH/3T3) were purchased from American Type Culture Collection (ATCC). A549 and HeLa cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO Company), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO2 at 37 °C. HepG2 and NIH/3T3 cells were maintained in DMEM
medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Company), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO₂ at 37 °C. BALB/c nude mice (6–8 weeks old) were purchased from the Animal Center of Zhejiang Academy of Medical Sciences. The use of animals was approved by the Animal Ethics Committee of Zhejiang Academy of Medical Sciences. Mice were housed in approved animal-care facilities on a 12 h light/dark cycle and given ad libitum access to food and water.

3. Synthesis of 1-(4-methylphenoxy)-2,4-dinitrobenzene and 1-[4-(bromomethyl)phenoxy]-2,4-dinitrobenzene. 1-(4-Methylphenoxy)-2,4-dinitrobenzene and 1-[4-(bromomethyl)phenoxy]-2,4-dinitrobenzene were synthesized according to the reported methods. Briefly, p-Cresol (1.08 g, 10 mmol), 1-fluoro-2,4-dinitrobenzene (2.80 g, 15 mmol) and anhydrous K₂CO₃ (3.12 g, 22.5 mmol) were dissolved in anhydrous DMF (100 mL), and the reaction mixture was heated to 80 °C for 4 hours under N₂ atmosphere. Then the mixture was poured to water (200 mL) and extracted by dichloromethane. The organic layer was washed with brine and dried with anhydrous Na₂SO₄, filtered and concentrated. Then the concentrated mixture was separated by silica gel column chromatography (hexyl hydride: dichloromethane) to obtain 1-(4-methylphenoxy)-2,4-dinitrobenzene (1.6 g, yield 58.6%). ¹H NMR (400 MHz, CDCl₃, δ): 8.74 (d, J = 2.7 Hz, 1H), 8.21 (dd, J = 9.3, 2.8 Hz, 1H), 7.20 (d, J = 8.5 Hz, 2H), 6.95 (dt, J = 7.6, 4.1 Hz, 3H), 2.32 (s, 3H).

1-(4-methylphenoxy)-2,4-dinitrobenzene (1.37 g, 5.0 mmol), N-bromosuccinimide (1.34 g, 7.5 mmol) and 2,2-azobisisobutyronitrile (1.23 g, 7.5 mmol) were dissolved in benzene (200 ml) and the mixture was heated under reflux in the dark under nitrogen for 24 hours. The solvent was removed by rotary evaporation. The crude mixture was separated by silica gel column chromatography (hexyl hydride: dichloromethane) to obtain 1-[4-(bromomethyl)phenoxy]-2,4-dinitrobenzene as a light-yellow solid (0.75 g, yield 42.5%). ¹H NMR (400 MHz, CDCl₃, δ): 8.85 (d, J = 2.7 Hz, 1H), 8.33 (dd, J = 9.2, 2.7 Hz, 1H), 7.57-7.45 (m, 2H), 7.21-6.99 (m, 3H), 4.52 (s, 2H).

was synthesized as our previous reports. Briefly, DEAEA (5 g, 29 mmol) and 2,2-azobisisobutyronitrile (0.024 g, 0.14 mmol) were mixed in a 25 mL flask and degassed at room temperature under nitrogen blanket for 30 min. Afterwards, polymerization was performed at 65 °C for 12 h. The viscous liquid was diluted in CH₂Cl₂ and precipitated in cold hexyl hydride. The process was repeated for three times and the product was dried under vacuum to obtain the product as a viscous solid. The PDEAEA samples with a molecular weight of 20 K and polydispersity of 1.6 determined by gel permeation chromatography (GPC) were obtained (4.2 g, yield 84%). ¹H-NMR (400 MHz, CDCl₃, δ): 4.1-3.9 (d, J = 5.8 Hz, 2H), 2.55 (dd, J = 32.6, 26.2 Hz, 6H), 2.25 (s, 1H), 1.64 (ddd, J = 61.7, 26.2, 19.2 Hz, 2H), 0.98 (t, J = 6.8 Hz, 6H).

5. Synthesis of cyanine5.5-labeled poly{N-[2-(acryloyloxy)ethyl]-N-[p-(2,4-dinitrophenoxy)benzyl]-N,N-diethyl ammonium chloride} (PADDAC⁵⁵⁵). PDEAEA functionalized with primary amines (PDEAEA-NH₂) was synthesized as our previous study and used directly. PDEAEA-NH₂ (100 mg) and fluorescent dye Cyanine 5-N-hydroxysuccinimide (Cy5.5-NHS) (1 mg) were dissolved in 2 mL DMF. The reaction mixture was stirred overnight at room temperature and then was added with 1-[4-(bromomethyl) phenoxy]-2,4-dinitrobenzene (353 mg, 1 mmol). After stirring for 24 h at 45 °C, the resulting solutions were dialyzed in DMF for 24 h and in sodium chloride solution for another 24 h using a dialysis membrane (molecular weight cut-off: 3,500 Da). The solution was lyophilized to obtained the product PADDAC⁵⁵⁵ as a dark blue solid (0.19 g, yield 61.5%). FITC-labeled PADDAC (PADDAC⁵⁵⁵) was synthesized by using fluorescein isothiocyanate as the same method described above.

6. Synthesis of the control polymer of N-[p-(2,4-dinitrophenoxy)benzyl] substituted linear polyethyleneimine (DNPB-PEI) and quaternized DNPB-PEI (DNPB-QPEI). Linear PEI (0.044 g, containing 1 mmol secondary amines) was reacted with 1-[4-(bromomethyl) phenoxy]-2,4-dinitrobenzene (0.37 g, 1 mmol) in 6 mL DMF at 45 °C for 24 h. 3 mL of the reaction mixture were dialyzed in DMF for 24 h and in methanol for 24 h using a dialysis membrane (Mw cut-off: 3500 Da). The solution was evaporated to obtain the product DNPB-PEI as a yellow solid. 1 mL of iodomethane
was reacted with the remnant mixture for 12 h. The resulting solutions were dialyzed in DMF for 24 h and in sodium chloride solution for another 24 h using a dialysis membrane (Mw cut-off: 3500 Da). The solution was lyophilized to obtained the product DNPB-QPEI as a yellow solid.

7. **Nuclear magnetic resonance (NMR).** NMR was measured on either Bruker ARX 400 NMR spectrometer or Agilent 600 MHz DD2 spectrometer. Chemical shifts signals (δ) were reported in parts per million (ppm) referenced relative to CDCl\textsubscript{3} (δ=7.26), DMSO (δ=2.50) or the tetramethylsilane Si(CH\textsubscript{3})\textsubscript{4} (δ=0). Multiplicities are reported as s(singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplet), m (multiplet), br (broadened).

8. **Gel permeation chromatography (GPC).** GPC analysis was conducted with a Wyatt liquid chromatography system using DMF with 0.01% LiBr or THF as the eluent at a flow rate of 0.8 mL/min at 35 °C. Narrow distributed polystyrenes with different molecular weights were used as standard samples.

9. **Transmission electron microscopy (TEM).** A 100-mesh copper grid covered with a carbon film was immersed in a polymer/DNA polyplex solution (20 μg/mL DNA) for 10 seconds and the excess solution was wicked off with filter paper. Samples were then negatively stained for 10 seconds by dipping in a saturated uranyl acetate aqueous solution. The morphology of polyplexes was visualized using a JEOL JEM-1010 TEM.

10. **High performance liquid chromatography (HPLC).** HPLC consisted of a 1525 binary pump, 2475 multi-λ-Fluorescence detector and 2998 photodiode array detector and a SunFireTM C\textsubscript{18} (4.6×250 mm, 5 μm) column. The mobile phase was using methanol:0.1% phosphorous acid solution = 3:1 (v/v) at a flow rate of 1.0 mL/min at 35 °C. The sample injection volume was 20 μL and the detection was performed at 275 nm.

11. **Particle size and zeta potential measurement of polyplexes.** The hydrodynamic diameter size (d\textsubscript{h}) and zeta potentials (ζ) of polyplexes were measured using Zetasizer Nano (Malvern Instrument Ltd. Inc., UK), equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at 25 °C. For particle size measurements, 100 μL
of polyplexes were used. Each sample was measured for three times at 25 °C. Data are presented as the means ± SD (n = 3). For zeta potential measurements, 100 μL polyplexes were diluted with 900 μL of 10 mM HEPES buffer (pH 7.4). Each sample was measured for 3 times at 25 °C.

12. Agarose gel electrophoresis. DNA condensation by polymers and its releasing by polyplex dissociation were evaluated by agarose gel retardation assay. Commercial loading buffer was used to mix with the polyplex solution at 1:5 (v/v). 20 μL of mixed solution was electrophoresed on the 1 wt% agarose gel with Tris acetate-EDTA running buffer at 120 V for 25 min. DNA band migration was visualized by staining with ethidium bromide (EB) and imaged via the fluorescence and chemiluminescence imaging system (Clinx Science Instruments Co., Ltd, China).

13. Preparation of polyplexes and lipopolyplexes. PADDAC was dissolved in HEPES buffer solution (10 mM, pH=7.4). Polymer solutions at various concentrations were added to the DNA solution of the same volume and vortexed for 10 seconds to produce PADDAC/DNA polyplexes at desired N/P ratios, defined as the molar ratio of nitrogen atoms in the polymer to phosphate units of the plasmid DNA. The polyplex solution was incubated at room temperature for 5 min before characterizations.

Lipid coated PADDAC/DNA i.e. LPADDAC/DNA, were prepared as our reported methods. For example, DOPE/CHEMS/DSPE-PEG with a mole ratio of 7.65/2/1.35 with a final concentration of lipid at 0.2 μmol/μg DNA were prepared as followings: DOPE (158 mg), CHEMS (27 mg), and DSPE-PEG (105 mg) were dissolved in 1 mL of chloroform and the solution was dried by rotary evaporator to obtain a thin lipid film. 125 μL of PADDAC/DNA polyplex solution (5 μg DNA, N/P = 10) was added to the lipid film, stirred overnight at room temperature, and sonicated for 1 min.

With increasing the amount of lipid coating, in particular from 0.05 to 0.2 μmol lipid per μg DNA, the zeta potential of LPADDAC/DNA lipopolyplexes was changing from neutral to slightly negative, indicating almost all polyplexes were coated with liposomes (Figure 4a). With 0.2 μmol lipid per μg DNA, all polyplexes would be coated assuredly, and the free amount of lipid was low and the lipopolyplexes did not need
further purification.

14. **Stability of polyplexes in serum and protein solution.** Polyplexes were diluted with culture medium containing different amount of FBS or with HEPES buffer (10 mM, pH=7.4) containing different amount of bovine serum albumin (BSA) and the mixture was incubated at 37 °C for 1 h. Afterwards, samples were taken out to measure their size and zeta potentials or the DNA release was evaluated by agarose gel retardation assay. Additionally, changes in the size of polyplexes incubated in HEPES buffer and culture medium containing 10% FBS at 37 °C were measured for 48 h.

15. **Cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** 5000 cells per well were seeded in the 96-well plate with 200 μL of 10% FBS-containing cell culture medium and incubated overnight. The cells were incubated with each vector in 10% FBS medium for 48 h. Afterwards, the medium was replaced with fresh culture medium containing 0.75 mg/mL MTT. After 3 h incubation, the medium was discarded and 100 μL of DMSO was added to dissolve the formazan crystals. The absorbance was detected at 562 nm using a microplate spectrophotometer (SpectraMax M2E, Molecular Device Inc. USA). The cytotoxicities of PEI and PADDAC polymer were tested similarly at the polymer concentrations ranging from 1 to 200 μg/mL in HeLa, A549, HepG2 and NIH/3T3 cells as the above-mentioned methods.

16. **Cell apoptosis measured by FITC-Annexin V and PI staining.** 200,000 HeLa cells per well were seeded in the 12-well plate with 2 mL cell culture medium containing 10% FBS and incubated overnight. LPADDAC/pTRAIL, PEI/pTRAIL and Lipo2000/ pTRAIL were added to the medium at a DNA concentration of 0.5 or 1 μg/mL. After 48 h incubation, morphological changes of apoptotic cells were imaged by light microscopy (OLYMPUS, BX51, Japan). Cells were rinsed with cold PBS, detached by trypsin, isolated and washed twice with cold PBS and then re-suspended in 1×Binding Buffer at a concentration of 1×10^6 cells/ml according to the manufacturer’s instructions (BD Pharmingen). 5 μL of FITC Annexin V and 5 μL PI were added to 100 μL of 1×10^6 cells suspension. The cell suspension was vortexed
gently and incubated for 15 min at room temperature in the dark, followed by adding 400 μL of 1× Binding Buffer to dilute the cells. The samples were analyzed by flow cytometry within 1 h.

17. **Subcellular distribution.** DNA was labeled with Cy5 (DNA<sup>Cy5</sup>) according to the manufacturer’s instructions (Mirus Bio). 100,000 HeLa cells per dish were seeded onto glass-bottom petri dishes with 1.0 mL of 10% FBS-containing culture medium and incubated for 24 h before use. The medium was replaced with 1.0 mL of fresh medium without FBS. Polyplex solutions (50 μL) labeled with different fluorescent dyes were added at a dose of 1 μg DNA per well. After timed incubation, the cells were incubated with Lysotracker green (Molecular Probes, Carlsbad, CA) at a concentration of 200 nM for 0.5 h to label the lysosomes, and the nuclei were stained with Hoechst 33342 (Molecular Probes, Carlsbad, CA) at a concentration of 10 μg/mL for 20 min. Cells were washed with PBS for three times before imaged by CLSM.

18. **Histological examination.** The tumors were fixed with 4% neutral buffered paraformaldehyde and embedded in paraffin. Tissue sections of 5 μm thick were mounted onto glass slides and stained with hematoxylin-eosin (H&E, Beyotime, China) and examined by light microscopy (OLYMPUS, BX51, Japan). Apoptotic events were also determined by the TdT-mediated dUTP nick end labeling (TUNEL) assay. Tissue sections were subjected to TUNEL staining using One Step TUNEL Apoptosis Assay Kit, according to the manufacturer’s protocol (Beyotime Biotechnology, China). Apoptotic cells were identified by positive TUNEL staining examined by CLSM.

19. **Western blotting analysis.** Freshly harvested tumors of each group were lysed in a cell lysis buffer (Beyotime, China). The protein contents were detected using BCA protein assay (Beyotime, China). About 40 μg of protein per well was loaded and separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore). The membrane was incubated first with 5% non-fat milk powder in Tris buffered saline with Tween-20 (TBST) to block the nonspecific binding sites and then incubated with primary antibodies (anti-TRAIL, 1:1000 and anti-caspase-3, 1:1000, Cell Signaling...
Technology). The membrane was rinsed in TBST and incubated with horseradish peroxidase-labeled goat antirabbit antibodies or horseradish peroxidase-labeled goat anti-mouse antibodies at 1:1000 dilution. Finally, the membrane was rinsed and visualized with electrochemiluminescence detection reagent (Beyotime, China).

20. **Statistical analysis.** All experiments were repeated at least three independent samples and each measurement were performed in triplicates. Data were presented as mean ± SD. Assignments to treatments and selections of fields of microscopic inspection were made at random. For individual comparisons, the two-tailed, unpaired student's t-test was performed. *P* < 0.05 was regarded as statistically significant.

**Supplementary Scheme, Figures and Table**

**Scheme S1.** Synthesis of a) the GSH-triggered charge-reversal polymer PADDAC, b) fluorescent dye-labeled PADDAC (PADDAC\textsubscript{Cy5.5}) and c) the control polymer PAPDAC.
Figure S1. The $^1$H NMR spectrum of 1-(4-methylphenoxy)-2,4-dinitrobenzene in CDCl$_3$.

Figure S2. The $^1$H NMR spectrum of 1-[4-(bromomethyl) phenoxy]-2,4-dinitrobenzene in CDCl$_3$. 
Figure S3. The $^1$H NMR spectrum of PDEAEA in CDCl$_3$.

Figure S4. The $^1$H NMR spectrum of PADDAC in deuterated DMSO.
Figure S5. The $^1$H NMR spectrum of PAPDAC in deuterated DMSO.

Figure S6. The $^1$H-NMR spectra of PADDAC (2 mg/mL) after reduction with GSH (10 mM) and self-catalyzed hydrolysis in deuterated DMSO/D$_2$O (4/1) at 37 °C.
Figure S7. a) Synthesis of the nonselective GSH-triggered degradable polymer DNPB-PEI and selective GSH-triggered degradable quaternary polymer DNPB-QPEI. 1H-NMR spectra of b) DNPB-PEI, c) DNPB-QPEI in deuterated DMSO.
Figure S8. GSH-triggered degradation of DNPB-PEI and DNPB-QPEI polymer. Responsive degradation of the polymer was monitored by using HPLC to detect the releasing \( p \)-hydroxymethylphenol (HMP). The selective response of DNPB-PEI and DNPB-QPEI to GSH, Cys, Hcy, NaSH (equivalent to 1 mM thiol) was incubated at pH 7.4 HEPES solution and 37 °C for 30 min.

Figure S9. GSH-triggered degradation of the control PAPDAC polymer and their polyplexes dissociation. a) Hydrodynamic diameter (\( d_h \)) and \( \zeta \) of PAPDAC/DNA polyplexes at different N/P ratios. b) Agarose gel electrophoresis of the polyplexes at different N/P ratios ranging from 2 to 12. c) Changes in \( d_h \) and \( \zeta \) of PAPDAC/DNA polyplexes (N/P=4) and d) DNA release after 1 h incubation with a serial of GSH concentrations.
**Figure S10.** The cytotoxicity assay of PADDAC and PEI polymer in HeLa, A549, HepG2 and NIH/3T3 cells.
Figure S11. Subcellular distribution of PADDAC\textsuperscript{Cy5.5}/DNA polyplexes imaged by CLSM. Cells were incubated with PADDAC\textsuperscript{Cy5.5}/DNA (1 μg/mL DNA) in serum free medium for 5, 15, 30, 60 and 120 min. PADDAC\textsuperscript{Cy5.5} was shown in red. Nucleus stained with Hoechst 33342 was shown in blue. Lysosome stained with Lyso Tracker Green was shown in green. Scale bars, 25 μm.
**Figure S12.** Agarose gel electrophoresis of LPADDAC/DNA lipopolyplex at different lipid/DNA ratio.

**Figure S13.** Agarose gel electrophoresis of a) PADDAC/DNA and b) LPADDAC/DNA polyplexes diluted in series concentration of BSA solution in HEPES buffer. Using naked DNA and polyplexes itself were used as control.
Figure S14. Changes in particle diameters of LPADDAC/DNA lipopolyplexes after incubated in HEPES buffer (10 mM, pH=7.4) and culture medium containing 10% FBS at 37 °C for 48 h.

Figure S15. Luciferase expression of LPADDAC/pLuci (0.2 μmol lipid per μg DNA, N/P = 10) in the presence of 0%, 10%, 50% and 100% FBS-containing culture medium in a) A549 and b) HepG2 cells.

Figure S16. In vitro TRAIL-induced apoptosis after cells transfected with LPADDAC/pTRAIL lipopolyplexes. MTT assay of cytotoxicity to HeLa, A549 and HepG2 cells mediated by LPADDAC/pTRAIL in the presence of 10% FBS medium.
Figure S17. The luciferase expression in HeLa xenograft tumor at 48 h after intratumoral injection of LPADDAC/pLuci, PEI/pLuci (N/P = 7) or Lipo2000/pLuci. n = 3, 10 μg pLuci per tumor in 50 μL HEPES buffer.

Figure S18. Antitumor activity of the LPADDAC/DNA lipopolyplexes in nude mice bearing intraperitoneal (i.p.) tumor. The mice were i.p. administered with LPADDAC/pTRAIL, PEI/pTRAIL and Lipo2000/pTRAIL (pTRAIL 2 mg/kg), PBS and PTX (10 mg/kg). The spleens were harvested from the mice of each group at the end of the experiment.
Figure S19. The fluorescence images of collected blood samples measured by Caliper IVIS Lumina II at timed intervals after i.v. injection of PADDAC<sub>Cy5.5</sub>/pTRAIL and LPADDAC<sub>Cy5.5</sub>/pTRAIL. PADDAC<sub>Cy5.5</sub>/pTRAIL or LPADDAC<sub>Cy5</sub>/pTRAIL polyplexes were i.v. injected at a dose of 40 μg pTRAIL (i.e. 2 mg/kg) per mouse (n = 4) in 200 μL HEPES buffer.

Table S1. Pharmacokinetic parameters of PADDAC<sub>Cy5.5</sub>/pTRAIL and LPADDAC<sub>Cy5.5</sub>/pTRAIL (n = 4, intravenously, eq. 2 mg/kg pTRAIL). Data were presented as mean ± SD.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>PADDAC&lt;sub&gt;Cy5.5&lt;/sub&gt;/pTRAIL</th>
<th>LPADDAC&lt;sub&gt;Cy5.5&lt;/sub&gt;/pTRAIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>h</td>
<td>0.54 ± 0.09</td>
<td>3.60 ± 0.27</td>
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<tr>
<td>AUC&lt;sub&gt;0-4&lt;/sub&gt;</td>
<td>Intensity/mL·h</td>
<td>98.25 ± 14.17</td>
<td>314.39 ± 78.75</td>
</tr>
<tr>
<td>MRT</td>
<td>h</td>
<td>0.68 ± 0.13</td>
<td>4.98 ± 0.46</td>
</tr>
</tbody>
</table>

T<sub>1/2</sub>, AUC<sub>0-4</sub>, and MRT refer to elimination half-time, area under curve and mean retention time respectively.

REFERENCES
