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Synthesis and Characterization of the Tumor Targeting Mitoxantrone-Insulin Conjugate

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Anticancer drugs have serious side effects arising from their poor malignant cells selectivity. Since insulin receptors highly express on the cytomembrane of some kind of tumor cells, using insulin as the vector was expected to reduce serious side effects of the drugs. The objective of this study was to evaluate the tumor targeting effect of the newly synthesized mitoxantrone-insulin conjugate (MIT-INS) with the drug loading of 11.68%. *In vitro* stability trials showed MIT-INS were stable in buffers with different pH (2-8) at 37°C within 120 h (less than 3% of free MIT released), and were also stable in mouse plasma within 48 h (less than 1% of free MIT released). *In vivo* study on tumor-bearing mice showed that, compared with MIT [75.92 μg·h/g of the area under the concentration-time curve (AUC) and 86.85 h of mean residence time (MRT)], the conjugates had better tumor-targeting efficiency with enhanced tumor AUC of 126.53 μg·h/g and MTR of 151.95 h. The conjugate had much lower toxicity to most other tissues with targeting indexes (TI^C) no larger than 0.3 besides good tumor targeting efficiency with TI^C of 1.67. The results suggest the feasibility to promote the curative effect in cancer chemotherapy by using insulin as the vector of anti-cancer drugs.

Key words: Mitoxantrone-insulin conjugate, Vector, Anti-cancer drug, Targeting

INTRODUCTION

Cytotoxic chemotherapy of cancers is limited by serious side effects that arise from toxicities to sensitive normal cells because the therapies are not selective for malignant cells. One strategy to improve the selectivity is to couple the therapeutics to ligands that recognize tumor-associated receptors. This increases the exposure of the malignant cells and reduces the exposure of normal cells, to the ligand-targeted therapeutics (Theresa, 2002). It has been found that some coding products of oncogenes are growth factors or their receptors, which destroy growth control mechanisms of normal cells to cause carcinomatous change (Von Horn *et al.*, 2001; Oka *et al.*, 2002). It may promote the curative effect by utilizing such growth factors, which lead to their receptors on the cytomembrane of tumor cells, as vectors of anticancer drugs (Hanafusa *et al.*, 2002; Huynh *et al.*, 2002). Recent researches showed higher expression and better affinity of insulin receptors on the cytomembrane of many kinds of tumor cells compared with normal cells (Tanaka *et al.*, 2002). Amir Jurtaran found that human hepatocellular carcinoma expressed a 1000-fold higher number of specific receptors for ^{123}I-Try-(A14)-insulin compared with normal liver tissue (Kurtaran *et al.*, 1995). Moreover, even when it is coupled with some compounds through covalent bonds, substantiated by experimentation, insulin still keeps the activity to combine with its receptor (Ou and Kuang, 2000).

Mitoxantrone (MIT) (Ehninger *et al.*, 1990), a cytotoxic anthracenedione derivative which intercalates with DNA and inhibits topoisomerase II, has been given clinical evidence of beneficial activity against breast cancer, hepatocarcinoma, lymphoma, leukaemia, and so on. Its primary side effect is cardiotoxicity (Koutions *et al.*, 2002). Improvement of the therapeutic index of MIT by using vectors may be a desirable treatment strategy. In this study, we have synthesized mitoxantrone-insulin conjugate (MIT-INS), and characterized the *in vitro* stability, *in vivo* biodistribution, tumor targeting property and pharmacokinetics in mice bearing H_{22} hepatocarcinoma. We hypo-

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thesize that MIT-INS will be targeted to solid tumors selectively.

MATERIALS AND METHODS

Chemicals and animals

MIT was supplied by Chongqing Carelife Pharmaceutical Co. Ltd. (Chongqing, P.R. China); insulin (28.1 $U{\cdot}mg^{-1}$) by Xuzhou Wanbang Biochemical Pharmaceutical Ltd. (Jiangsu, P.R. China); *N*-hydroxybenzotriazole (HOBT) by GL Biochem (Shanghai) Ltd. (Shanghai, P.R. China); 1-Ethyl-3-(3-dimethyllaminopropyl)-carbodiimide hydrochloride (EDC) by Sigma. Ametantrone was presented as a gift from National Cancer Institute (USA). All other chemicals and reagents used were of analytical grade obtained commercially. Female Kunming mice weighing 18-22 g were received from Sichuan Industrial Institute of Antibiotics (P.R. China) and maintained on a stock diet and water ad libitum. Ascitic H_{22} cells were provided by Sichuan Industrial Institute of Antibiotics (P.R. China). The H_{22}-Hepatocarcinoma was obtained by implanting subcutaneously $2{\times}10^6$ ascitic H_{22} cells in right flanks of the mouse. It was found in our preliminary experiments that the repeatability was not good enough if the tumor weight was less than 1 g and the growth rate of tumor was inconsistent, therefore experiments were not initiated until consistent growth rate and a minimum tumor weight of 1 g was achieved. Since the experiment was done within 48 h, there was no significant change in body weight and tumor size of mice between treated groups and untreated group.

Instruments

IR spectra were recorded on a Nicolet 200SXV spectrophotometer. ^{1}H-NMR spectra were run at 400 MHz on a Brucker-Ac-200 spectrometer and the chemical shifts were in ppm downfield from tetramethylsilane. MS spectra were obtained with an Autospec-Ultima ETOF FAB+ magnet instrument. Melting point was taken on an YRT-3 melting point apparatus. Ultraviolet spectral measurements were performed on a Shimadzu UV-2201 spectrophotometer with 1 cm quartz cells.

Buffer solutions

Buffer A-H: phosphate buffer (0.05 M phosphoric acid, 0.05 M sod. phosphate dibasic, 0.05 M phosphate monobasic, and 0.05 M sod. hydroxide were mixed to give pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 7.4 respectively).

Buffer I: ascorbic acid buffer (0.010 mol citric acid and 0.238 mol ascorbic acid were mixed with 100 mL distilled water to give pH 3.0).

Buffer J: 0.2 M ammonium acetate buffer (0.2 M ammonium acetate was adjusted to pH 2.0 using sulfuric acid).

HPLC analysis

The HPLC system consisted of an SPD-10A variable UV-VIS detector, a Model FCV-12AH column-switching valve, and a set of Model LC-10AT liquid chromatograph including two pumps, a manometric module and a dynamic mixer from Shimadzu. The mobile phase consisted of methanol/buffer J (48/52) solution, which was filtered through 0.45 μm membrane filter before use. A Shimpack ODS column (150×4.6 mm, 5 μm) was eluted with the mobile phase at a flow rate of 1.0 mL/min. The eluate was monitored by measuring the absorption at 599 nm at sensitivity of AUFS 0.01 at 30°C. The Class VP V5.0 software was employed for the data analysis. The retention time of MIT was 5.75 min.

Determination of MIT content

HPLC was used to determine the MIT content of MIT-INS (Zhang and Zhang, 1999). The mixture of MIT-INS solution (0.5 mL), 6 M HCl (0.25 mL) and buffer I (0.1 mL) was hydrolyzed at 95°C for 20 min to entirely release MIT. The hydrolyzed solution was centrifuged at 9000 g for 10min and filtered through a membrane filter (0.45 μm). The filtrate (20 μL) was analyzed by HPLC.

Column switching HPLC analysis

A GEL-CN12S11 column (50×4.6 mm, 15 μm, YMC CO., Ltd., Kyoto, Japan) was used as pre-column. Purified water was used as washing mobile phase at a flow rate of 1.0 mL/min with 3 min of switching time. Other HPLC conditions were the same as those mentioned above. The retention time of ametantrone and MIT was 8.35 min and 9.86 min, respectively.

Calibration of MIT in various biological media

Stock solution of MIT was prepared in distilled water (100 μg/mL). A portion of the stock solution was diluted to obtain series of working solution of MIT (0.2, 1.0, 5.0, 10.0, 15.0, 20.0, 35.0 μg/mL). Blood, heart, lung, liver, spleen, kidney and tumor were obtained from mice bearing H_{22} hepatocarcinoma. Fresh blood was poured into a test tube containing heparin (0.1 mg/mL, heparin/blood) and centrifuged at 9000 g for 10 min to obtain plasma. Other tissues were homogenized separately and diluted with water (30 w/w%). To a 0.5 g portion of the above plasma and homogenates mixed with buffer solution I (0.1 mL), 6 M HCl (0.25 mL) and internal standard of ametantrone solution (10 μg/mL, 0.1 mL), were added each of the working solution of MIT (0.1 mL) and appropriate volume of methanol to make the final volume of 5 mL, which provided standard solution of MIT in concentration of 4, 20, 100, 200, 400 or 600 ng/mL and of ametantrone in concentration of 200 ng/mL, respectively, in various biological specimen.

Standard or blank solution (5 mL) was mixed on a vortex mixer for 2 min, centrifuged at 9000 g for 10min and filtered through a membrane filter (0.45 μm). The filtrate (1 mL) was analyzed by column switching HPLC as described in previous section and a calibration curve was constructed.

Preparation of MIT di-succinic ester

To the solution of MIT (517 mg, 1 mmol) in 20 mL of dimethylformamide, succinic anhydride (200 mg, 2 mmol) and triethylamine (0.5 mL) were added in portions with stirring at 65°C for 24 h under the protection of nitrogen. The reaction mixture was evaporated under reduced pressure to remove the solvent. The residue was dissolved in 0.5 M NaOH (20 mL) at 0°C and adjusted to pH 4.5 with 2 M HCl. The resultant precipitate was collected by centrifugation and dried in vacuum to obtain mitoxantrone di-succinic ester (MDSE) in 95% overall yield. UV λ_{peak} 615, 669 nm; mp 124.6-125.6°C; IR (KBr) 1720 (COOR) cm^{-1}; FABMS *m/z* 643 (M^+); ^{1}H-NMR (TMS, DMSO) δ 2.45 (t, J = 6.4 Hz, 4H, 12-CH_2, 16-CH_2), 2.61 (t, J = 6.4 Hz, 4H, 13-CH_2, 17-CH_2), 3.36~3.41 (m, 4H, 21-CH_2, 25-CH_2), 3.51~3.56 (m, 8H, 11-CH_2, 15-CH_2, 14-CH_2, 18-CH_2), 3.64 (t, J = 6.4Hz, 4H, 20-CH_2, 24-CH_2), 7.16 (s, 2H, 6-H, 7-H), 7.71 (d, J = 4.4Hz, 2-H, 3-H), 10.59 (brs, 2H, 1-OH, 4-OH), 13.52 (brs, 2H, 22-COOH, 26-COOH).

Preparation of MIT-INS

To the solution of MDSE (400 mg), EDC (500 mg) and HOBT (250 mg) in 30 mL of 0.01 M HCl, the solution of insulin in 20 mL of 0.01 M HCl was added with stirring at 20°C for 72 h under the protection of nitrogen. The reaction mixture was adjusted to pH 4.5, and the precipitate was discarded by centrifugation. The supernatant was adjusted to pH 5.3, kept at 4°C for 12 h, and centrifuged to obtain the resultant precipitate. The precipitate was dissolved in distilled water, dialyzed for 3 days to remove lower molecular substances, and lyophilized to obtain MIT-INS in 66% yield. UV δ_{peak} 615, 669 nm; IR (KBr) 1650 (COOH) cm^{-1}; MIT content 11.68%.

pH Stability and plasma stability *in vitro*

A solution of MIT (50 μg/mL) was incubated in buffer A, B, C, D, E, F and G at 37°C for 120 h under the protection of nitrogen. A solution of MIT (100 μg/mL) was also incubated in 30% plasma solution of buffer H at 37°C for 48 h. At a predetermined time interval, a 20 μL portion of the solution was removed, and the concentration of MIT was analyzed by HPLC as described previously.

Biodistribution in tumor-bearing mice *in vivo*

The Sichuan University animal ethical experimentation committee, according to the requirements of the National Act on the use of experimental animals (P.R. China), approved all procedures of the studies *in vivo*. MIT and MIT-INS were injected through caudal vein of the mice. For each preparation and sampling time point, five mice were treated with a single dose equivalent to 2 mg/kg of MIT (0.2 mL/10 g body weight). The dose of MIT-INS was 17 mg/kg (MIT content 11.68%), which was equivalent to 2 mg/kg of MIT.

At the predetermined time, blood samples were collected from the ocular artery directly after eyeball removal and treated as described previously to obtain plasma samples. The animals were dissected and each tested organ was removed, including heart, lung, liver, spleen, kidney and tumor. Every organ samples were accurately weighed and homogenized. To a 0.5 g portion of these plasma and homogenates, buffer solution I (0.1 mL), 6 M HCl (0.25 mL), internal standard of ametantrone solution (10 μg/mL, 0.1 mL) were added. These mixtures were hydrolyzed at 95°C for 20 min, and were added appropriate volume of methanol to make the final volume of 5 mL when cooling to obtain sample solutions, which were treated and analyzed by column switching HPLC as described previously.

Pharmacokinetic analysis

Plasma and tissue concentration data of MIT and MIT-INS obtained *in vivo* were pooled to provide mean concentration data. The pharmacokinetics of MIT and MIT-INS was described as tow-compartment open model. The pharmacokinetic parameters in plasma and organs were obtained as follows. The area under the concentration-time curve (AUC) was calculated using the linear trapezoidal rule and extrapolated to infinity by dividing the last measurable concentration by the elimination rate constant. Mean residence time (MRT) was calculated by statistic moment program. Targeting index (TI^C), overall targeting efficiency (TE^C) and relative overall targeting efficiency (RTE^C) of MIT-INS were calculated to evaluate the tumor selectivity of the conjugate compared with MIT (Wang *et al.*, 2002), where

$$TI^C = (AUC_{0\to\infty})_{MIT-INS}/(AUC_{0\to\infty})_{MIT}$$

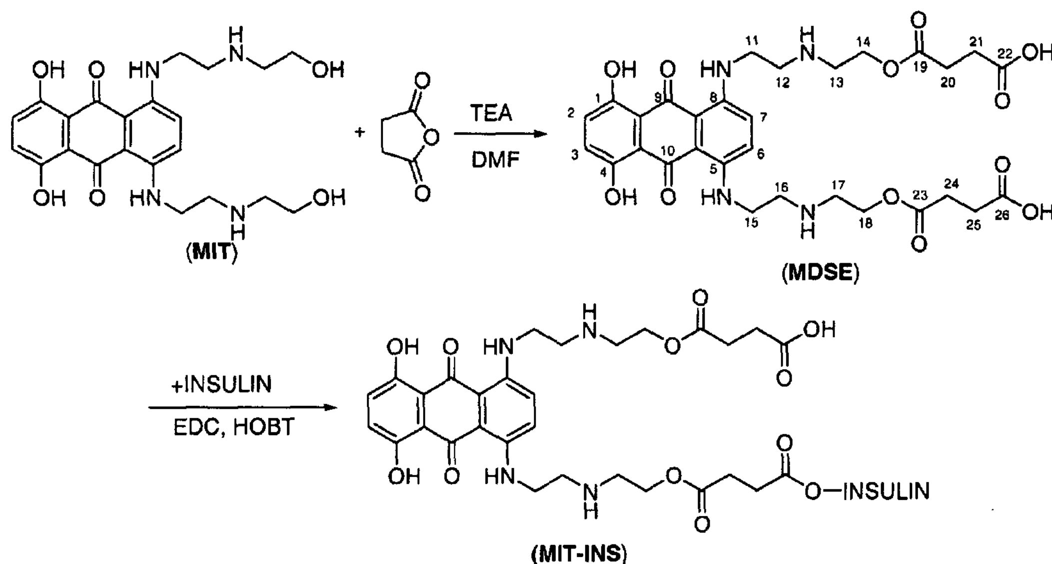
$$TE^C = \{(AUC_{0\to\infty})_i/\sum_{i=1}^{n}(AUC_{0\to\infty})_i\}\times 100\%$$

$$RTE^C = \{(TE^C_{MIT-INS}-TE^C_{MIT})/TE^C_{MIT}\}\times 100\%.$$

RESULTS AND DISCUSSION

Preparation of MIT-INS

MIT has hydroxyl and secondary amino groups which insulin can be coupled with directly or through a spacer. In the experiments, we found that it is difficult to couple MIT with insulin directly under moderate conditions, which is to prevent insulin from denaturation, so we chose succinic



Scheme 1. Synthesis of mitoxantrone-insulin conjugate

anhydride as a spacer. The schematic synthetic route was shown in Scheme 1. The first step was to synthesize MDSE as the intermediate and then MIT-INS as the product. The structure of MDSE was proved by IR, ^{1}H-NMR, MS and UV. IR spectrum showed an absorption peak of ester at 1720 (COOR) cm^{-1}, which suggested the formation of the ester. MS spectrum showed the molecular weight of the ester was 644, which suggested the ester was MIT di-succinic ester instead of MIT mono-succinic ester, because the molecular weight of MIT and succinic acid was 444 and 100 respectively. ^{1}H-NMR spectrum showed the existence of two carboxyl groups in the ester molecule, which further substantiated the structure of MIT di-succinic ester. The structure of MIT-INS was also determined. IR spectrum showed an absorption peak of ester at 1650 (COOH) cm^{-1}, which suggested the carboxyl group remained in the molecule of MIT-INS. MIT content (11.68%) showed the molar ratio of MIT to insulin (molecular weight 5800) within the conjugate was approximately 1.2:1.

pH Stability and plasma stability *in vitro*

For tumor-specific drug delivery, it is crucial for the conjugate to keep intact during transportation after administration, such as undergoing blood circulation, distribution and re-distribution in tissues of different pH values, until it arrives at the target. In this case, the conjugate can be combined to insulin receptor on tumor cells and then enter cell through phagocytosis (Ou and Kuang, 2000), where it is hydrolyzed by enzymes secreted by lysosomes to release MIT. Then, it is necessary to trial the stability *in vitro* of MIT-INS in solutions of different pH value and plasma to lay the foundations for further studies *in vivo*. We adopted the solution of 30% mouse plasma in the trial

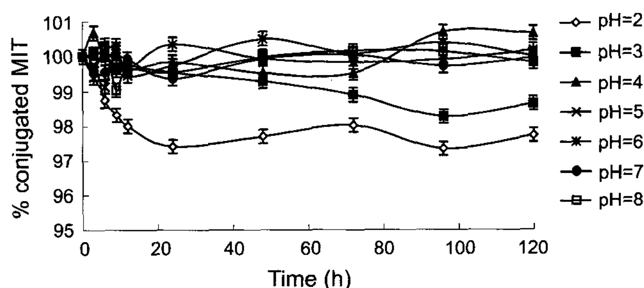


Fig. 1. Time course of stability of MIT-INS in different pH phosphate buffers at 37°C. Data are mean±SE (n=3).

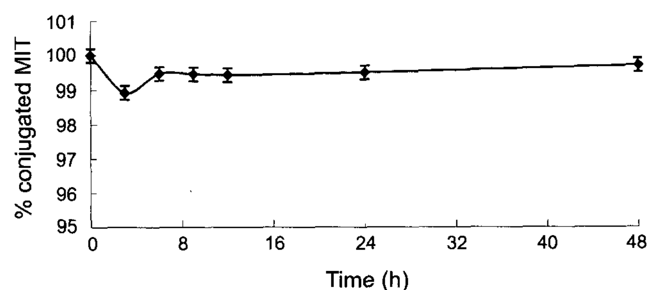


Fig. 2. Time course of stability of MIT-INS in 30% plasma at 37°C. Data are mean±SE (n=3).

because of the high protein binding capacity of MIT. It was shown in Fig. 1 that the conjugates were stable in the solutions of pH 2-8 at 37°C within 120 h with less than 3% of free MIT released and was especially stable in the solutions of pH 4-8. It was shown in Fig. 2 that the conjugates were also stable in 30% mouse plasma solution at 37°C within 48 h with less than 1% of free MIT released. These data suggested the good stability of the conjugate

in vitro and indicated its suitability for further studies *in vivo*.

Biodistribution and pharmacokinetic analysis *in vivo*

Table I shows pharmacokinetic parameters of MIT-INS and MIT in plasma and tumor. Table II shows drug concentration versus time in plasma and tumor after *i.v.* injection in mice. Table III shows TE^C of MIT-INS and MIT. TI^C and RTE^C, shown in table IV, were calculated to evaluate targeting effect of MIT-INS compared with MIT.

As shown in these tables, there were significant differences of biodistribution and pharmacokinetics in tumor-bearing mice between MIT-INS and MIT (AUC or MRT, when α=0.05, P<0.01). Compared with MIT (75.92 μg·h/g of AUC and 86.85 h of MRT), the tumor targeting efficiency of MIT-INS was improved, with enhanced tumor AUC of 126.53 μg·h/g and MTR of 151.95 h. The overall drug targeting efficiency (TE^C) of tumor was enhanced from 0.8% to 17.05%, leading to a prolonged $t_{1/2}$ value in the tumor from 60.19 h to 105.30 h. The targeting index and relative overall targeting efficiency of tumor were 1.67 and 20.20 respectively. The initial concentration of drug in tumor was doubled. It is known that higher concentration and retention time of the drug in targeted tumor tissue were in favor of eradication the disease, especially with higher initial concentration. In the mean time, higher drug concentration in blood stream and lower drug diffusion to other tissues ensured drug supply to tumor, because of the high blood accommodate of tumor tissue in the body. The tables also showed that MIT-INS kept in blood with higher concentration and longer circulation time compared with MIT. The AUC and $t_{1/2}$ value of MIT-INS in plasma were 29.36 μg·h/g and 296.76 h, much larger than those of MIT (3.81 μg·h/g and 0.36 h). Longer circulation of MIT-INS in blood stream may attribute to its larger molecular size resulting in a decreased glomerular filtration rate of the kidney. On the other hand, the mechanisms of endocytosis between MIT-INS and MIT may be also different, where the former were mediated by insulin receptors (Kurtaran *et al.*, 1995). From this point of view, MIT-INS, with longer retention time, higher drug concentration in tumor, was better than MIT in the targeting therapy of tumor.

Table III showed that TE^C value of MIT group is tree times higher than MIT-INS group against lung. This was caused by higher distribution amount and lower elimination rate of MIT in the lung compared with MIT-INS. It means MIT may have higher toxicity than MIT-INS to normal lung cells.

Table IV showed that the values of TI^C and RTE^C in tumor were much larger than those of other tissues (heart, liver, spleen, lung and kidney). The TI^C of tumor (1.67) was higher than 1 and those of other tissues (no higher than 0.3) were far less than 1, which meant that MIT-INS was far less inclined to distribute to heart, liver, spleen,

Table I. Pharmacokinetic parameters of MIT-INS and MIT in plasma and tumor. Tow-compartment open model: $C = Ae^{-\alpha t} + Be^{-\beta t}$. $K_{21} = (A\beta + B\alpha)/(A + B)$, $K_{10} = \alpha\beta/K_{21}$, $K_{12} = \alpha + \beta - K_{21} - K_{10}$, $t_{1/2\alpha} = 0.693/\alpha$, $t_{1/2\beta} = 0.693/\beta$, $AUC = A/\alpha + B/\beta$, $MRT = \int_0^\infty tCdt/\int_0^\infty Cdt$, $t_{1/2} = 0.693MRT$

Parameter	$Plasma_{MIT-INS}$	$Plasma_{MIT}$	$Tumor_{MIT-INS}$	$Tumor_{MIT}$
A (ug/g)	15.71	1.65	2.01	0.88
á (1/h)	8.30	9.17	3.15	13.99
B (ug/g)	0.21	0.11	0.87	0.90
â (1/h)	0.01	0.03	0.01	0.01
$t_{1/2á}$ (h)	0.08	0.08	0.22	0.05
$t_{1/2â}$ (h)	88.96	23.76	100.49	58.65
K_{21} (1/h)	0.12	0.58	0.96	7.07
K_{10} (1/h)	0.54	0.46	0.02	0.02
K_{12} (1/h)	7.65	8.16	2.18	6.91
AUC (μg·h/g)	29.36	3.81	126.53	75.92
MRT (h)	428.23	0.52	151.95	86.85
$t_{1/2}$ (h)	296.76	0.36	105.30	60.19

Table III. Overall targeting efficiency (TE^C) of MIT-INS and MIT (%)

Tissue	Heart	Liver	Spleen	Lung	Kidney	Blood	Tumor
MIT-INS	11.42	8.15	21.01	20.98	17.43	3.94	17.05
MIT	11.22	2.17	13.69	66.00	6.08	0.04	0.80

Table IV. Targeting evaluation of MIT-INS compared with MIT

Tissue	Heart	Liver	Spleen	Lung	Kidney	Blood	Tumor
TI^C	0.08	0.30	0.12	0.02	0.23	7.71	1.67
RTE^C (%)	0.02	2.76	0.53	-0.68	1.87	97.04	20.20

Table II. Drug concentration in plasma and tumor after *i.v.* injection in mice (μg/g, MIT/tissue)

Time (h)		0.083	0.25	0.5	1	2	4	8	12	24	48
Plasma	MIT-INS	8.20	1.99	0.66	0.34	0.17	0.18	0.17	0.19	0.16	0.17
	MIT	0.88	0.26	0.15	0.10	0.10	0.09	0.08	0.07	nd	nd
Tumor	MIT-INS	2.34	2.01	1.11	1.08	0.83	0.87	0.84	0.75	0.72	0.64
	MIT	1.17	0.92	0.89	0.89	0.87	0.86	0.85	0.76	0.65	0.52

nd: not detected

lung and kidney and thus had much lower toxicity to all these tissues.

In conclusion, the better targeting efficiency of MIT-INS may mainly come from two aspects: firstly, higher drug concentration in blood stream and lower drug diffusion to other tissues due to larger molecular size of the conjugate; secondly, difference of the mechanisms of endocytosis between MIT-INS and MIT, where MIT-INS mediated by insulin receptor. There may be other mechanisms, which contribute to the results, to be further explored. In short, the result of this study may present a potential method to promote the curative effect in cancer chemotherapy by using insulin as the vector of anti-cancer drugs.

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