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Pharmacokinetics and disposition study of calf thymus DNA in rats by applying ^{3}H-labeling method

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ABSTRACT

A tritium(^{3}H)-labeling method with high specificity was established to investigate the pharmacokinetics and disposition of the calf thymus DNA (ctDNA) in rats. The plasma pharmacokinetics, tissue distribution, mass balance and excretion were characterized in SD rats, respectively. Rats were injected i.v. with radiolabeled ctDNA with the dose of 40 μCi/kg in each independent experiment. ^{3}H-labeled ctDNA was eliminated rapidly in plasma, with the half-life estimated from 9 to 13 h and preferentially accumulated in liver and lung, its concentration in all the tissues investigated decreased to very low level after 24 h. ctDNA exhibited 80.8% accumulative recovery, excretion of radiolabel in urine and bile was nearly complete by 72 h, which shown as the main excretion pathways, and the total recovery of excretion reached 77.9% within three days. In conclusion, ctDNA was rapidly eliminated in plasma and would not accumulate in tissues, parent ctDNA and its radioactive metabolites can be recovered almost completely in schedule time. All the results indicated that the in vitro use of ctDNA is safe and will not bring out potential risk.

1. Introduction

DNA immunoadsorption column is developing rapidly in recent years as a new blood purification engineering technology with high specificity, which can selectively remove the endogenous pathogenic factors in blood of patients by antigen–antibody immune response or physical and chemical effects, so that blood is purified and endogenous diseases are alleviated largely [1,2]. Calf thymus deoxyribonucleic acid (ctDNA) is one of the absorptive materials used in vitro leprosy hemodialysis, which clear up leprosy bacillus by physical adsorption effectively. However, the possibility that some ctDNA fall off from the column carrier in the application process thus go into blood and consequently, go into circulatory system with returning blood purified to the body of patient, limits its practical use for this unexpected risk for health potentially. To overcome the limitation, it is indispensable to evaluate pharmacokinetic property of ctDNA before its therapeutic use.

During the past few decades, a number of nucleic acids were discovered as promising tools in research and clinical practice [3–6], but most pharmacokinetic studies were focused on small fragment nucleic acids, predominantly the antisense oligonucleotides, with about twenty nucleotide units polymerized (20-mer) [7,8], which exhibit inhibitory effect by hybridizing with their complementary sequences in mRNA and thus "knocking down" their expressions [9,10]. Unlike these low molecular weight analogues, ctDNA is the mixture composed of large amounts of nucleic acid molecules without identical structure and molecular weight, so it is quite difficult to characterize its pharmacokinetic profile and quantitate it in vivo by using conventional approaches. Virtually, there is no information in the literature concerning the pharmacokinetics of this kind of macromolecular compounds. Given these features of ctDNA, radioisotope labeling maybe the preferred optional means to achieve the goal [11].

The objective of the present study was to investigate pharmacokinetics and disposition of ctDNA after administration in rats, so as to provide reliable guidance from the viewpoint of safety for its practical application as carrier material.

2. Materials and methods

2.1. Chemicals and reagents

ctDNA was provided by Jianfan Biotechnology Company (Zhuhai, China), isopropanol was purchased from Sinopharm Chemical Reagent Company, purified water was prepared with MilliQ® Ultrapure water purification system in XenoBiotic Laboratories, Inc-China (Nanjing, China), ULTIMA GOLD™ scintillation fluid was purchased from PerkinElmer Life Sciences (Boston, MA). All other reagents were of analytical grade.

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2.2. Radiolabeling of ctDNA

Radiolabeling experiment was conducted by Shanghai Institute of Applied Physics, Chinese Academy of Sciences (Shanghai, China), isotope exchange method was used to synthesize the radiolabeled ctDNA. In brief, certain amount of ctDNA was dissolved in phosphate buffer, 5% $PdO/BaSO_4$ was added as catalyst, exchange reaction was conducted in 50 °C water bath, ultimately, stirring. Tritiated product was obtained after removing the unstable tritium. The specific activity of the final preparation was 1.2 mCi/mg. ^{3}H-labeled ctDNA was stored at 4 °C until use.

Radiolabeled ctDNA was mixed together with "cold" ctDNA for animal experiments. Sufficient ^{3}H-labeled ctDNA dilution with alcohol as solvent was mixed with unlabeled sample, and then was added to 5% glucose solution, followed by heating at 50 °C and stirring until the solution was mixed uniformly. During the administration process, ^{3}H-labeled ctDNA solution was kept in vortexing with the concentration of 10 μCi/mL.

2.3. Animals

Male and female Sprague-Dawley rats weighing between 200 and 220 g were purchased from Center of Experimental Animals of Academy of Military Medical Sciences (Beijing, China). The animals were housed in polypropylene cages (one rat per cage) and allowed access to food and water ad libitum. All studies were in compliance with the Guidelines for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee in XBL-China.

2.4. Plasma pharmacokinetic study

Rats were randomly divided into four dose groups as detailed in Table 1. ^{3}H-labeled ctDNA was administered once a day or consecutively for seven days by i.v. injection via the lateral tail vein for single-dose and multiple-dose administration, respectively. Before dosing, each rat in group 1 for blood collection had a jugular cannula implanted, after administration, whole blood was collected at every scheduled sampling time point in tubes containing heparin sodium as the anticoagulant. Blood samples were immediately frozen on dry ice and centrifugated with the speed 3000 rpm in 4 °C for 10 min to separate plasma. All samples were stored at −20 °C until analysis.

2.5. Tissue distribution

After i.v. injection, six animals in group 2 were euthanized with excess CO_2 at each time point in Table 1. Blood was drawn by cardiac puncture and the normal tissues, including heart, liver, spleen, lung, kidney, brain, body fat, intestine, muscle, testis/ovary, were taken, weighed and collected with animal carcasses remained.

2.6. Mass balance and excretion

Recovery of radiolabel in urine, feces, carcasses and cage wash was assessed after single-dose injection. Compared with mass balance experiment, rats in group 4 assigned to excretion study received a biliary ducts cannula and the urine, feces and bile were collected in metabolic cage, respectively. Time intervals for sample collection of the above two experiments are shown in Table 1.

2.7. Bioanalytical methods

Concentrations of all samples were determined on the basis of the ctDNA-derived radioactivity quantitated by liquid scintillation counter (Tri-Carb 3110TR, Perkin Elmer Life Sciences). Feces was homogenized after adding water:isopropanol (1:1) mixed liquor, oxygen combustion of the homogenate was carried out by using biological sample oxidizer (OX-501, R.J. Harvey), ULTIMA GOLD™ scintillation fluid was used to trap the ^3H_2O generated from combustion. The tissue samples and carcasses were dissolved by adding KOH solution. Plasma, urine, bile and cage wash were mixed directly with liquid scintillation fluid.

The scintillation counter was operated in the background subtract mode and samples were counted for a minimum of 2 min, counted numbers per minute (CPM) was converted to disintegrations per minute (DPM) automatically.

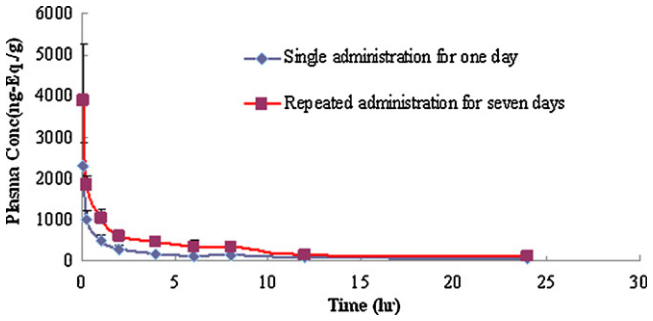

Fig. 1. Plasma ctDNA equivalent concentration–time profiles after intravenous injection of 5 mg/kg dose. Each point represents the average of six animals.

2.8. Data processing and analysis

Data were analyzed and pharmacokinetic parameters were estimated by using WinNonlin (Version 5.3, Pharsight) and non-compartmental model, based on the mean radioactivity in plasma. Data are given as mean ± SD, mean values were considered to be significantly different when $p < 0.05$ by using a Student's *t*-test.

3. Results

3.1. Plasma pharmacokinetics

The plasma concentration–time distribution profile was shown in Fig. 1. Compared with single administration, concentrations determined at each time point for repeated administration were all relatively higher, however, it could infer that ctDNA was rapidly cleared in plasma after intravenous injection irrespective of the dosing frequency. Twelve hours after administration while single-dosing injection was applied, the concentration of ctDNA was dramatically declined to 76 ng equiv./g (a number of ng of ctDNA equivalent per gram of plasma or tissue samples containing), corresponding figure was 136 ng equiv./g for repeated administration. Half-lives of both the frequencies of administration were 13 ± 6.0 and 9 ± 2.7 h, respectively (Table 2).

3.2. Tissue distribution

As shown in Fig. 2, radioactivity in most tissues (except intestine) reached peak level at 0.25 h after intravenous injection. The highest mean radioactivities were detected in liver and lung, followed by kidney and spleen, concentrations in body fat and genital glands were minimal among all the tissues, but there was no significant difference ($p > 0.05$) between different tissues. However, for all the tissues investigated the concentration of ctDNA decreased to very low level without any exception at 24 h after administration (lower than 1 ng equiv./g), suggesting that ctDNA will not accumulate in tissues and organs, in addition, concentration of ctDNA in

Table 1
Pharmacokinetic experimental design and sample collection.

Study purpose	Number and gender	Dose level (μCi/kg)	Time points and intervals
PK	6 male 6 female	40	0.033, 0.25, 1, 2, 4, 6, 8, 12 and 24 h
Biodistribution	9 male 9 female	40	0.25, 2 and 24 h
Mass balance	3 male 3 female	40	Urine/feces: 0–4, 4–8, 8–12, 12–24 h
Excretion pathway	3 male 3 female	40	Bile: 0–8, 8–24, 24–48, 48–72 hUrine/feces: 0–8, 8–24, 24–48, 48–72 h

Table 2
Plasma pharmacokinetic parameter estimates for ctDNA after intravenous injection of 5 mg/kg ($n = 6$). Data represent average ± standard deviation.

Parameter[a]	AUC_{0-24} (ng equiv. h/g)	$AUC_{0-\infty}$ (ng equiv. h/g)	$T_{1/2}$ (h)	MRT (h)	CLZ (mg/kg)/(h ng/g)	V_{ss} (mg/kg)/(ng/g)
Single administration	3571 ± 851	4514 ± 1133	13 ± 6.0	6 ± 0.74	0.00115 ± 0.000288	0.015933 ± 0.006028
Repeated administration	7601 ± 1861	8838 ± 2451	9 ± 2.7	6.06 ± 1.1	0.0006 ± 0.0002	0.0062 ± 0.001934

[a] Estimates are for total radioactivity and, thus, include both parent drug and metabolites.

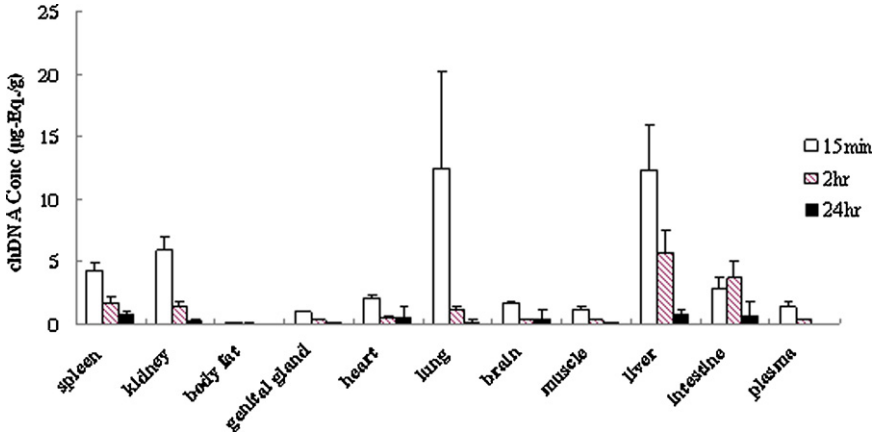

Fig. 2. Biodistribution of ctDNA. Data are expressed as mean ± SD of six animals at each time point. Statistical comparisons between different tissues were performed by Student's *t*-test.

plasma was quite low and decreased along with the time, being in agreement with the results obtained from plasma PK.

3.3. Mass balance

The urine and feces samples were collected within 24 h after intravenous injection for mass balance study. Excretion of ^{3}H-labeled ctDNA in urine and feces experienced a significant increase along with the time, the total recovery of radioactivity in urine was 24.4% within 24 h, corresponding Figure of feces was 15.92%, a little lower than the former one (Table 3). It is worth noting that radiolabel residue of ^{3}H-ctDNA in rat carcass remained approximately 40% at the last time point, which was almost equal to the summation of recovery in urine and feces. Therefore, the accumulative radiolabel recovery calculated from various samples reached 80.8% at 24 h after administration, including the radioactivity in cage wash.

Table 3
Mass balance excretion of radiolabel residue of ^{3}H-ctDNA over 24 h after a single 5 mg/kg intravenous injection. Each data represents the average of six animals.

Sample	Time intervals, recovery of radioactivity (%)					In total (%)
	0–4	4–8	8–12	12–24	Accumulation	
Urine	12.83	3.9	2.13	5.55	24.4	80.8
Feces	0.024	0.077	6.46	9.36	15.92	
Cage wash	–	–	–	–	1.53	
Carcass	–	–	–	–	38.96	

3.4. Excretion

Excretion experiment was conducted roughly following the same procedure as mentioned in mass balance study. It can be observed that the total recovery of excretion reached 77.9% within the scheduled time, the excretion percents obtained from bile and urine gradually rose, and the former one reached 32.07% at the last time point, which exhibited the maximal amount of excretion (Fig. 3). The sum of excretion of radiolabel in urine and bile was nearly about 60% within 72 h, much more than the excretion percents of radiolabel in feces (less than 20%), this result was not surprising if we consider that ctDNA is water-soluble as one of

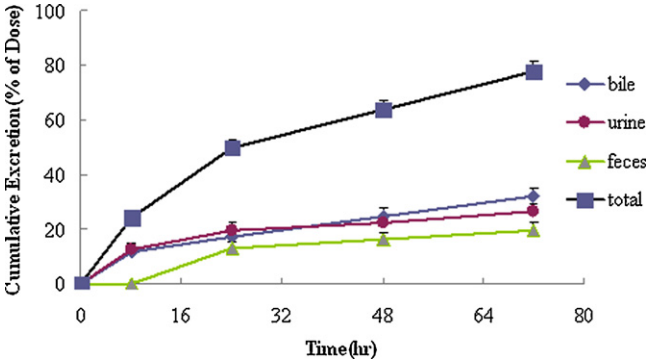

Fig. 3. The curve of cumulative excretion for ctDNA by three pathway after intravenous injection of 5 mg/kg dose. Each point represents the average of six animals.

nucleic acids, as well as large amounts of tritium atoms (^{3}H) labeled in ctDNA were transferred to hydrones in body.

4. Discussion

At present, almost all the nucleic acids developed as promising drugs are micromolecular oligonucleotides, with identical chemical structure and relatively low molecular weights. Unlike these compounds, ctDNA in this study were composed of a large number of nucleic acid molecules with varying molecular weights, so it is hard to determine quantitatively and conduct pharmacokinetic studies for them by using conventional methods due to their uncertain structures, especially the mass balance and excretion study, in which ctDNA will be metabolized and degraded to metabolites with lower molecular weights and more complicated composition in vivo. Thus, this problem could be solved only by applying isotope labeling method to "label" ctDNA and using radioactive isotope detection [12]. The common methods used for isotope labeling are chemical synthesis, biological chemistry and isotope exchange method, and the most widely used nuclides including ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{76}Br, ^{125}I [13]. In general, tritium labeling is relatively easy to carry out and the ^{3}H-labeled product possesses relatively higher specific activity (S_A, the number of decays per unit time per amount of substance), thus tritium was selected as the tracer to label ctDNA in this study [14,15].

Various modes of administration have been applied in the pharmacokinetic studies of nucleic acid drugs in literature, including i.v. injection, subcutaneous injection, oral administration and so on [16–18], but actually ctDNA did not display therapeutic effect in vivo. In its practical application, the blood of patients was put through the absorptive carriers where ctDNA was located to remove virus, so it is quite apparent that if ctDNA fall off during the process of treatment, it would go into blood directly. This is why only the i.v. administration was used in this study while other administration pathways were not considered.

Be similar to traditional chemicals, multiple dosing administration was applied in most pharmacokinetic studies for oligonucleotides to investigate the possibility of accumulation [19,20]. Although ctDNA was not used in vivo, it is possible that blood dialysis required to be conducted for several times rather than once according to specific needs in its in vitro application, thus ctDNA maybe potentially fall off and go into the body with many times. In order to obtain more comprehensive information of pharmacokinetics, multiple dosing administration was also investigated in this paper. Results indicated that ctDNA was rapidly cleared in plasma regardless of the dosing frequency. It is worth noting that its pharmacokinetic profile does not meet the typical compartment model, we infer that this is because the plasma concentration determined was actually converted from the total radioactivity, which was the sum of parent ctDNA and its radioactive metabolites, owing to the complex composition and difficulty in metabolite identification for ctDNA, in other words, it is the mixture composed of complicated compositions rather than original ctDNA that were determined in pharmacokinetic study. Meanwhile, this is perhaps the reason why the half life of ctDNA in plasma was longer than most oligonucleotides though it can be cleared quickly as well.

Results of the study showed that more ctDNA distributed in liver and lung and tissue distributions were in a rapid decrease after i.v. injection, which is inconsistent with the distribution feature of oligonucleotides [21,22]. The total radiolabel recovery collected within 24 h was 80.8%, considering the fact that ctDNA did not accumulate in tissues and organs significantly, the incomplete recovery may be due to the following two aspects: the collection period may not be long enough (slow excretion), the excreta was not be completely collected and some radioactive sample was missing, in addition, radioactivity may be lost for excreta was eliminated via another route such as sweat evaporation [23]. In fact, a considerable amount of tritium atoms originally labeled in ctDNA were transferred to hydrones by tritium(^{3}H)–hydrogen(H) exchange, which could account for the recovery of radioactivity in carcass was up to 38.96% in 24 h after dosing, thus actually most of the tritium were located in tritiated water molecules rather than ctDNA, being indicative of that not much ctDNA remained in rat body.

Compared with mass balance study, the time interval and total time for collection of various kinds of excreta in excretion experiment were designed longer, so as to clarify the excretion pathways and amount of excretion through each pathway of ctDNA more clearly [24].

5. Conclusion

In summary, the present study showed clearly that ctDNA was rapidly eliminated in plasma after i.v. administration and would not accumulate in the main tissues. Twenty-four hours after administration plasma and tissue concentration all decreased to very low levels and total radiolabel recovery reached more than 80%, results of excretion study indicated that ctDNA was almost entirely cleared through feces, urine and bile, and the latter two were the main excretion pathways. Therefore, according to the above data, it is apparent that ctDNA will be quickly removed and will not reside in body for long periods, even though some of it fall off and go into the body in practical application for in vitro therapy. This study for the first time provided reliable pharmacokinetic guidance for ctDNA and proved that it could be used safely without bringing out serious potential risk and side effect.

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