

Synthesis and preclinical pharmacological evaluation of a novel ^{99m}Tc -shikonin as a potential tumor imaging agent

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Abstract Shikonin was isolated from Ratanjot pigment then the obtained shikonin was well characterized. This study is aimed to optimize radiolabeling yield of shikonin with ^{99m}Tc with respect to factors that affect the reaction conditions such as shikonin amount, $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ amount, reaction time and pH of the reaction mixture. In vitro stability of the radiolabeled complex was checked and it was found to be stable for up to 6 h. Biodistribution studies showed that, ^{99m}Tc -shikonin accumulate in tumor sites with higher T/NT than other currently available $^{99m}\text{Tc}(\text{CO})_3\text{-VIP}$, ^{99m}Tc -nitroimidazole analogues and ^{99m}Tc -polyamine analogues indicating that shikonin deliver ^{99m}Tc to the tumor sites with a percentage sufficient for imaging and can overcome many drawbacks of other radiopharmaceuticals used for tumor imaging.

Keywords Shikonin · Extraction · Ratanjot pigment · ^{99m}Tc · Tumor

Introduction

Shikon (*Lithospermum erythrorhizon* Sieb. et Zucc, Chinese name: Zicao), a herbal plant, has been used in complementary and alternative medicine for thousands of years. Its dried root possesses therapeutic properties evidenced by it inhibits inflammation and enhances wound healing [1, 2]. The antitumor activity of shikon has been observed and confirmed by western science [1, 3]. Studies demonstrated that shikon arrests the cell cycle at G1/G0 and induces apoptosis in tumor cells [4].

Several bioactive components in Shikon have been identified and purified including β,β -dimethylacrylalkannin, acetylshikonin, β -acetoxylvaleryl alkannin, shikonin (SK) and β -hydroxyisovalerylalkannin [5]. In particular, SK (Fig. 1) has been characterized as a key component, and studies demonstrated that it has antiinflammatory and antitumor activities [6]. SK inhibits tumor cell proliferation in different cancer cell lines [7–9].

Decreased tissue oxygen tension (hypoxia) in tumor cells is thought to result in increased resistance of solid tumors to radiotherapy and chemotherapy.

The development of noninvasive imaging radiopharmaceutical targeted to tumor hypoxia represents a solution for the wide drawback of the invasive methods to diagnose hypoxia. In the development of hypoxia imaging agent, nitroimidazole analogues, which are enzymatically reduced and accumulated in hypoxic regions, have received great attention as hypoxia imaging agents. Several ^{99m}Tc labeled nitroimidazole analogues have been reported, however they showed some defects like, lower tumor uptakes and slow blood clearance [10–15].

Recently, ^{18}F -Fluoromisonidazole (^{18}F FMISO) [16, 17], $^{99m}\text{Tc}(\text{CO})_3\text{-VIP}$ and ^{99m}Tc -polyamine analogues has been used to evaluate tumor hypoxia [18]. But, the short half life

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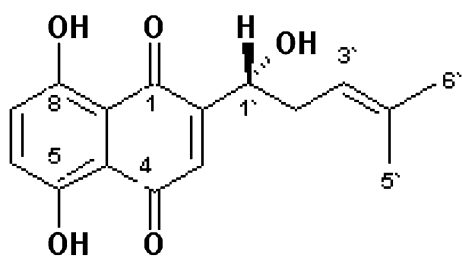


Fig. 1 Shikonin structure

and high cost of the [^{18}F] isotope restrict the wide application of [^{18}F]FMISO in clinical nuclear medicine while $^{99\text{m}}\text{Tc}(\text{CO})_3\text{-VIP}$ and $^{99\text{m}}\text{Tc}$ -polyamine analogues showed low percent tumor hypoxia uptake ranging from 0.5 to 1 [19, 20].

Many studies showed that shikonin exerted antitumor effects by inhibiting cancer cell growth [10], inducing apoptosis [21] and inhibiting DNA topoisomerase III activity, [7, 22, 23] antitelomerase activity [24] and antiangiogenesis. [25] Ratanjot pigment was used as a source for shikonin and the real source of Ratanjot was recently reported to be applied to about 15 plant species belonging mostly to family Boraginaceae including *Lithospermum erythrorhizon* [26]. Phytochemical investigation of authentic ratanjot hexane solubles was performed by many researchers and shikonin was reported as one of its components beside its ester derivatives [26, 27]. In this study shikonin was obtained from Ratanjot hexane extract after being subjected to alkaline hydrolysis and characterized with different analytical techniques. Shikonin was labeled with $^{99\text{m}}\text{Tc}$ and factors affecting the labeling yield were studied in details. $^{99\text{m}}\text{Tc}$ -shikonin complex was biologically investigated in mice bearing solid tumor.

Experimental

General

Melting points were determined on Branstead Google electrothermal melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on Perkin Elmer FT-IR Spectrum BX Spectrometer at cm^{-1} scale using KBr discs. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ were carried out on Bruker AC 500 MHz and JEOL 300 MHz Spectrometer using TMS as internal stander and chemical shift values were recorded in ppm on δ scale. The $^1\text{HNMR}$ data were represented as follow: chemical shifts, multiplicity (*s*. single, *d*. doublet, *t*. triplet, *q*. quartet, *b*. broad, *m*. multiplet) and number of protons. $^{13}\text{CNMR}$ data were represented as chemical shifts and type of carbon. Thin layer chromatography was performed on precoated (0.75 mm) silica gel

GF $_{254}$ plates (E. Merck, Germany). Visualization was performed by illumination with UV light source (254 nm). Standard shikonin was purchased from Biomol (Plymouth Meeting, PA, USA).

Extraction and hydrolysis of shikonin ester derivatives

Ratanjot powder was purchased from Canadian store cranberry lane (0.5 kg) and was extracted with *n*-hexane using soxhlet until no more pigment was removed. The extract was evaporated to give red viscous oil (6 g). The pigment (0.5 g) was shaken with aqueous 2 N-sodium hydroxide at RT for one hour. The blue solution was filtered, cooled in ice, and acidified with dilute acetic acid. The red suspension was extracted with chloroform (3×150 ml). The phenolic hydrolysis product was obtained as a red solid by evaporation of the chloroform solution. Repeated crystallization from ether-petroleum yielded shikonin as red prisms (100 mg), m.p. 147–148° (lit., 147°), R_f 0.44 (*n*-hexane/ethyl acetate 80:20 v/v) identical with authentic sample of shikonin.

Shikonin: red prisms; R_f 0.44 (*n*-hexane/ethylacetate 80:20 v/v); $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ 1.68 (3 H, *s*, $\text{CH}_3\text{-5}'$), 1.78 (3 H, *s*, $\text{CH}_3\text{-6}'$), 2.38, 2.66 (2 H, *m*, H-2'), 4.93 (1 H, *dd*, $J = 4.5, 3$ Hz, H-1'), 5.23 (1 H, *m*, H-3'), 7.18 (1 H, *s*, H-3), 7.20 (2 H, *s*, H-6, 7), 12.45 (1 H, *s*, OH-5), 12.60 (1 H, *s*, OH-8); $^{13}\text{C-NMR}$ (CDCl_3 , 500 MHz) δ 18.1 (C-5'), 26.0 (C-6'), 35.7 (C-2'), 68.3 (C-1'), 111.5 (C-10), 112.0 (C-9), 118.5 (C-3'), 131.7 (C-3), 132.3 (C-7), 132.4 (C-6), 137.4 (C-4'), 151.5 (C-2), 164.9 (C-8), 165.5 (C-5), 179.8 (C-4), 180.6 (C-1); IR ν_{max} (cm^{-1}): 3436 (OH), 1696 (C=O), 1610 (C=O), 1452 (C=C). Comparison of NMR and IR data with that of an authentic sample of shikonin showed the two to be indistinguishable.

Labeling procedure

Exactly 600 μg of shikonin, dissolved in 1 ml N_2 -purged distilled water, were separately transferred to evacuated penicillin vials. Exactly 50 μg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ was added to each vial along with different amounts of 0.1 N NaOH or 0.1 N HCl to adjust pH in a range of 1–7. Then, 1 ml of freshly eluted $^{99\text{m}}\text{TcO}_4^-$ (400 MBq) was added to each vial. The reaction mixtures were left at room temperature for 30 min. The same procedure was repeated with varying $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ amounts (10–70 μg), varying shikonin amounts (500–750 mg), and different reaction times (5–450 min).

Radiochemical purity of $^{99\text{m}}\text{Tc}$ -shikonin complex

The percent labeling yield of the labeled $^{99\text{m}}\text{Tc}$ -shikonin complex was determined by using ascending paper chromatographic technique. Strips of Whatman No. 1 paper

chromatography of 13 cm long and 0.5 cm wide were marked at a distance of 2 cm from the lower end and lined into sections 1 cm each up to 10 cm. A spot from ^{99m}Tc -shikonin complex solution was applied using hypodermic syringe, and then the strip was developed in an ascending manner in a closed jar filled with N_2 gas to prevent oxidation of the labeled shikonin spot. The developing solvents were acetone for developing one paper and saline (0.9 % NaCl) for developing another one. After complete development, the strips were dried and cut into fragments 1 cm each. Then the sections were counted in a NaI(Tl) γ -ray scintillation counter.

It was further confirmed by a Shimadzu HPLC system, which consists of pumps LC-9A, Rheodyne injector and UV spectrophotometer detector (SPD-6A) operated at a wavelength of 520 nm. Chromatographic analysis was performed by injection of 10 μl from the reaction mixture of ^{99m}Tc -shikonin into a reversed-phase column (Lichrosorb RP18, 4 mm \times 250 mm; 5 μm). The isocratic solvent system used was $\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{CH}_3\text{COOH}:\text{Et}_3\text{N}$ (630:370:3:3, v/v). Chromatography was performed at a flow rate of 0.5 ml/min, injection volume of 10 μl , pressure of 140 bar, and a column temperature of 23 $^\circ\text{C}$. Fractions of 0.5 ml were collected separately using a fraction collector up to 12 ml and counted in a well-type γ -scintillation counter.

In vitro stability of ^{99m}Tc -shikonin complex

The in vitro stability of ^{99m}Tc -shikonin complex was investigated as a function of time up to 8 h post labeling.

Induction of tumor in mice

The parent tumor line (Ehrlich Ascites Carcinoma) was withdrawn from 7 days old donor female Swiss Albino mice and diluted with sterile physiological saline solution to give 12.5×10^6 cells/ml. Exactly 0.2 ml solution was then injected intramuscularly in the right thigh to produce a solid tumor. The animals were maintained till the tumor development was apparent (10–15 day) [28].

Biodistribution study

The study was approved by the animal ethics committee, Labeled Compound Department, and was in accordance with the guidelines set out by the Egyptian Atomic Energy Authority. Biodistribution of ^{99m}Tc -shikonin was carried out in a group of male Albino Swiss mice weighted 20–25 g. Each animal was injected in the tail vein with 0.2 ml solution containing 100 KBq of ^{99m}Tc -shikonin. The animals were sacrificed by cardiac puncture post anesthesia at 0.25, 1, 4 and 6 h post-injection (p.i.).

Various organs, tissue and tumors were excised following sacrifice, washed with normal saline, dried and the radioactivity associated with each organ and tissue was determined using a flat type NaI(Tl) counter. The weights of each organ and tumor were also determined by using an analytical balance. The percent injected dose (%ID) in various organs, tissue and tumor were calculated from the above data and expressed as %ID per gram (%ID/g) of organ/tissue. The total uptake in blood, bone and muscles were calculated by assuming that 7, 10 and 40% of the body weight are constituted by this organs/tissue, respectively [29, 30]. Differences in the data were evaluated with the Student *t* test. Results for *P* using the two-tailed test are reported and all results are given as mean \pm SEM. The level of significance was set at *P* < 0.05.

Results and discussion

Radiolabeling of ^{99m}Tc -shikonin complex

Using ascending paper chromatographic technique, The radiochemical purity of the formed ^{99m}Tc -shikonin complex was checked by using acetone as a developing solvent where free $^{99m}\text{TcO}_4^-$ was moved with the solvent front ($R_f = 1$) while other species (reduced hydrolyzed ^{99m}Tc colloid and ^{99m}Tc -shikonin complex) remained at the point of spotting. The percentage of the reduced hydrolyzed- ^{99m}Tc was calculated using saline as developing solvent where reduced hydrolyzed- ^{99m}Tc colloid remained at the origin ($R_f = 0$) while free $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -shikonin complex migrate to the top of the paper. The percent of ^{99m}Tc -shikonin complex were determined as follows:

$$\% \text{labeling yield} = 100 - \left(\% \text{free } ^{99m}\text{TcO}_4^- + \% \text{reduced hydrolyzed } ^{99m}\text{Tc} \text{ colloid} \right)$$

An HPLC radiochromatogram (Fig. 2) showed two peaks, one at fraction number 2, which corresponds to $^{99m}\text{TcO}_4^-$ whereas the second peak was collected at fraction number 10, which corresponds to ^{99m}Tc -shikonin complex, which was found to coincide with the UV signal. Nearly, 98 % of the injected activity in the HPLC was recovered as collected activity.

Effect of shikonin concentration

As shown in Fig. 3, at low shikonin concentration (500 μg) the yield was small and equal to 85.3 ± 0.9 %. These low labeling yields were due to the shikonin concentrations being insufficient to form complex with all of the reduced

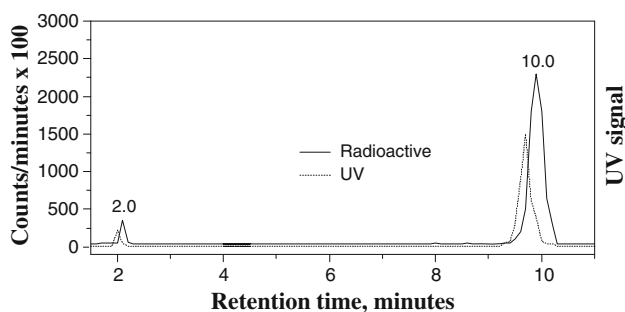


Fig. 2 HPLC radiochromatogram of ^{99m}Tc -shikonin complex

technetium so, the remaining reduced ^{99m}Tc was converted to reduced hydrolyzed technetium colloid ($13.5 \pm 2.1\%$). Increasing the shikonin concentration lead to higher labeling yield and the maximum yield of $96.5 \pm 4.75\%$ was achieved at $600\ \mu\text{g}$. Increasing the shikonin concentration over the optimum values, nearly did not affect on the labeling yield.

Effect of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ concentration

For contributing most of ^{99m}Tc to increase the labeling yield of shikonin, $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ is the best reducing agent for reduction of ^{99m}Tc from (VII) to lower valence state, which facilitates its chelation by shikonin. The effect of the amount of stannous chloride was summarized in Fig. 4. At $10\ \mu\text{g}$ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, the labeling yield of ^{99m}Tc -shikonin was low ($12.6 \pm 0.3\%$) due to $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ concentration was insufficient to reduce all pertechnetate so the percentage of $^{99m}\text{TcO}_4^-$ was high ($84.6 \pm 2.2\%$). The labeling yield was significantly increased by increasing the amount of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ from 10 to $50\ \mu\text{g}$, at which maximum labeling yield of $96.5 \pm 4.75\%$ was obtained. By increasing the amount of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ above the optimum concentration value, the labeling yield decreased again. This may be due to the fact that most of the ligand molecules were consumed in the formation of complexes, so the

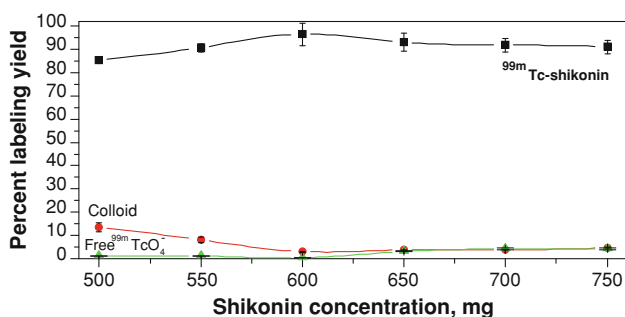


Fig. 3 Effect of shikonin concentration on the percent labeling yield of ^{99m}Tc -shikonin complex

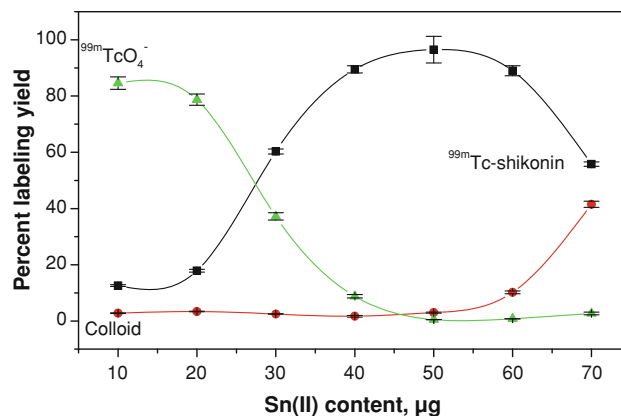


Fig. 4 Variation of the radiochemical yield of ^{99m}Tc -shikonin as a function of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ concentration

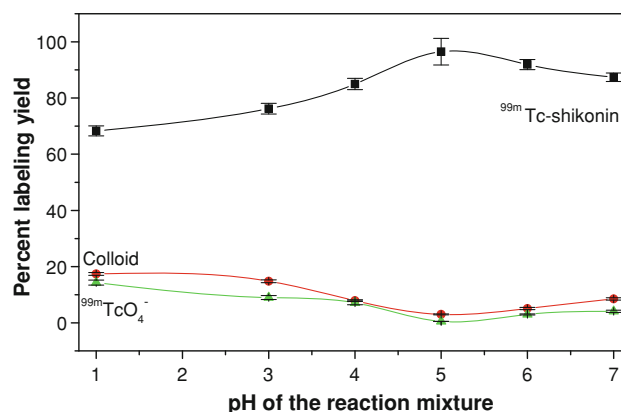


Fig. 5 Effect of pH of the reaction medium on the formation of ^{99m}Tc -shikonin complex

pertechnetate is reduced to insoluble technetium (IV) $\text{TcO}_2 \cdot x\text{H}_2\text{O}$ in the absence of ligand [31] or due to the fact that the excess amount of stannous chloride leads to the formation of stannous hydroxide colloid $\text{Sn}(\text{OH})_3$ ($41.5 \pm 1.1\%$ at $70\ \mu\text{g}$ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) in basic medium [32, 33].

Effect of pH of the reaction mixture

Figure 5 clearly shows that, the labeling yield of ^{99m}Tc -shikonin complex was increased with increasing the pH of the reaction mixture where at pH 1 the labeling yield was $68.3 \pm 1.8\%$ and at pH value equal to 5, the maximum labeling yield ($96.5 \pm 4.75\%$) was obtained where at this pH value shikonin combined all the reduced ^{99m}Tc . When the pH value was increased towards the neutral pH, the percent labeling yield was decreased again which reached to $87.4 \pm 1.5\%$ at pH 7.

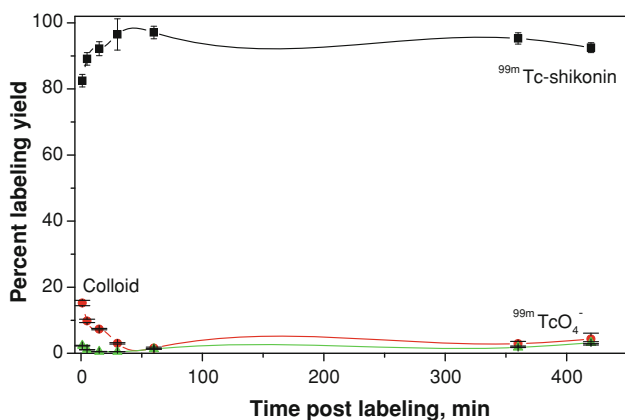


Fig. 6 ^{99m}Tc-shikonin yields versus reaction time

Effect of reaction time and stability

Figure 6 describes the labeling yield of ^{99m}Tc-shikonin complex at different reaction times.

The labeling yield of ^{99m}Tc-shikonin complex increased with time till reaching 96.5 ± 4.75 % at 30 min reaction time. It remained constant (~96 %) even when the reaction time was extended to 6 h.

Biodistribution

Biodistribution of ^{99m}Tc-shikonin in solid tumor bearing mice was found to be greatest in blood, heart and lung (16.8 ± 0.2, 6.0 ± 0.1 and 4.1 ± 0.2 %, respectively) at 15 min post injection and lowest in left leg and Stomach

(1.06 ± 0.03 and 0.1 ± 0.0, respectively) (Table 1). Most of radioactivity was excreted through the urinary pathway where the activity in urine reached to 42.8 ± 2.2 % at 6 h post injection. The activity uptake of ^{99m}Tc-shikonin in the right thigh (inoculated) was greater than that of left one at all time intervals. The maximum T/NT for ^{99m}Tc-shikonin (T/NT = 8.63) was significantly higher than that of the currently available ^{99m}Tc(CO)₃-VIP, nitroimidazole analogues and ^{99m}Tc-polyamine analogues (T/NT = 0.5–1) indicating that shikonin deliver ^{99m}Tc to the tumor sites with a percentage sufficient for imaging and can overcome all drawbacks of other radiopharmaceuticals for tumor imaging.

Conclusion

In this study shikonin was isolated from Ratanjot pigment and then well characterized using different analytical techniques. Shikonin was labeled with ^{99m}Tc using simple and instantaneous method. The labeling of shikonin with ^{99m}Tc was optional in the following reaction mixture: 600 µg shikonin, 50 µg SnCl₂·2H₂O, pH 5 and 30 min reaction time. The formed complex was stable for time up to 6 h. ^{99m}Tc-shikonin was found to be highly localized in tumor sites (T/NT = 8.63) where shikonin was considered an ideal victor to carry ^{99m}Tc to the tumor cells. In conclusion, ^{99m}Tc-shikonin could be used as a new non nitroimidazole hypoxic imaging agent in the future and this study demonstrates a hopeful approach for cancer imaging.

Table 1 Biological distribution of ^{99m}Tc-shikonin complex in solid tumor bearing mice

Organs & body fluids	% Injected dose/g organs and body fluids at different time post injection (h)			
	0.25	1	4	6
Blood	16.8 ± 0.2	10.0 ± 0.1	6.0 ± 0.2	1.3 ± 0.0
Bone	1.2 ± 0.1	1.5 ± 0.1	1.1 ± 0.2	0.6 ± 0.2
Liver	3.4 ± 0.2	3.7 ± 0.2	4.1 ± 0.2	6.5 ± 0.2
Lung	4.1 ± 0.2	4.2 ± 0.2	3.9 ± 0.2	2.5 ± 0.1
Heart	6.0 ± 0.1	4.5 ± 0.1	2.0 ± 0.5	1.6 ± 0.1
Stomach	0.1 ± 0.0	0.2 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
Intestine	3.1 ± 0.2	3.9 ± 0.2	4.0 ± 0.2	4.7 ± 0.1
Kidney	8.4 ± 0.2	13.0 ± 0.8	10.3 ± 0.2	7.8 ± 1.2
Urine	19.2 ± 1.4	25.0 ± 2.8	36.5 ± 3.2	42.8 ± 2.2
Spleen	2.37 ± 0.3	4.0 ± 0.02	4.9 ± 0.3	2.7 ± 0.1
Left leg	1.06 ± 0.03	0.67 ± 0.02	0.65 ± 0.1	0.66 ± 0.01
Right leg	3.53 ± 0.0.2	4.94 ± 0.1	5.61 ± 0.09	5.68 ± 0.0.2
T/NT	3.33	7.37	8.63	8.61

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