Distribution of Pirarubicin in Human Blood

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We investigated the distribution and stability of pirarubicin in human blood obtained from 12 healthy volunteers. The distribution of pirarubicin into blood cells showed marked temperature- and concentration-dependencies and the Arrhenius plot for pirarubicin uptake in blood was biphasic. Therefore, pirarubicin appears to be taken up into blood cells by a carrier-mediated system. Pirarubicin was mainly enzymatically metabolized to pirarubicinol in blood cells, but pirarubicin was not metabolized into doxorubicin in either blood or plasma. On the other hand, in plasma, pirarubicin was degraded to unknown inactive compounds instead of pirarubicinol. It is therefore suggested that blood cells serve to protect against the degradation of pirarubicin into inactive compounds in blood. Accordingly, when the monitoring of pirarubicin and its active metabolites is carried out in patients, both blood and plasma must be frozen immediately after blood collection.

Keywords pirarubicin; pirarubicinol; doxorubicin; human blood; distribution; stability

Introduction

Pirarubicin (THP), synthesized by Umezawa *et al.*,[1] has higher clinical efficacy against several solid tumors, acute leukemia and malignant lymphoma,[2,3] and a lower incidence of side effects (*e.g.* cardiotoxicity, alopecia and gastrointestinal disorder) compared with other anthracyclines such as daunorubicin (DNR) and doxorubicin (ADR).[2-4] For these reasons, THP is widely used. We previously reported the pharmacokinetics of THP in pediatric patients and indicated that there were large interindividual variations in the blood to plasma concentration (B/P) ratio of THP, *i.e.* in the uptake of THP by blood cells.[5]

The objective of our study is to investigate the distribution of THP in human blood obtained from healthy volunteers. In addition, the stability of THP in blood or plasma was investigated.

Materials and Methods

Chemicals Pirarubicin hydrochloride (Therarubicin® for injection) was supplied by Meiji Pharmaceutical Co., Ltd. (Tokyo, Japan). Pure THP, pirarubicinol (THP-OH) and DNR (internal standard for HPLC) standards were also provided by Meiji Pharmaceutical Co., Ltd. The ADR standard was provided by courtesy of Kyowa Pharmaceutical Co., Ltd. (Tokyo, Japan). Analytical grade reagents and solvents were purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Samples Fresh human blood was obtained from twelve healthy consenting volunteers (six male and six female; mean age, 23 years). The volunteers had refrained from alcohol and medication intake for at least 1 d prior to blood donation. Blood was collected in heparinized syringes. In the blood experiment, the blood obtained (20 ml) was preincubated for a definite time (about 20 min) at 0, 2, 10, 15, 20, 30 and 37 °C in the incubator without shaking. The THP solution dissolved in distilled water was added to the incubated blood to a final concentration of 0.5% (v/v). The THP concentration in the incubation medium was 0.319 μM (200 ng/ml), except in the concentration-dependency experiments where concentrations ranged up to 7.97 μM (5000 ng/ml). Two ml of the blood were then removed into centrifuge tubes at 0.33, 5, 15, 30, 60, 120 and 240 min. An aliquot (1 ml) of the blood was kept for determination of blood drug concentrations, while the remainder was centrifuged at 14000 rpm for 1 min to obtain the plasma samples. The blood and plasma samples obtained were immediately frozen at −80 °C and stored until the time of assay. In the plasma experiment, a freshly obtained plasma sample (10 ml) was preincubated for a definite time (about 20 min) at 37 °C. After THP solution (0.5% (v/v) of plasma) was added to the incubation medium, aliquots (0.5 ml) of the plasma were sampled at the same times as in the blood experiment. The plasma samples were then treated in the same manner as the blood samples.

Assay Procedure The THP, ADR and THP-OH concentrations in blood and plasma were determined by HPLC as reported previously.[5]

Hematocrit Value and White Blood Cell (WBC) Counts Determination of the hematocrit (Ht) value of each blood sample was performed in the usual microhematocrit capillary tubes. WBC counts in each sample were determined on a hematocytometer.

Statistical Analysis The data are expressed as mean ± S.D. Comparisons between the two groups were performed by means of an unpaired t-test corrected for unequal variance. A statistically significant difference was defined as a p value of 0.05 or less.

Results

Effect of Temperature on Distribution of THP in Blood We examined the time courses of THP B/P ratios in normal human blood. As shown in Fig. 1, the THP B/P ratio at 37 °C reached a steady state at about 60 min after the addition of THP, and THP B/P ratios were increased with a prolongation of incubation period at all temperatures. The B/P ratio of 37 °C showed higher values than those at other temperatures from 15 min after the addition of THP, but the ratio decreased gradually from 60 min. As shown in Fig. 2, the Arrhenius plot for THP uptake by blood cells was biphasic, and the apparent transition temperature was 20 °C. The relationship between the B/P ratio of samples incubated with 0.319 μM of THP for 60 min at 37 °C and the WBC counts of 11 subjects was shown in

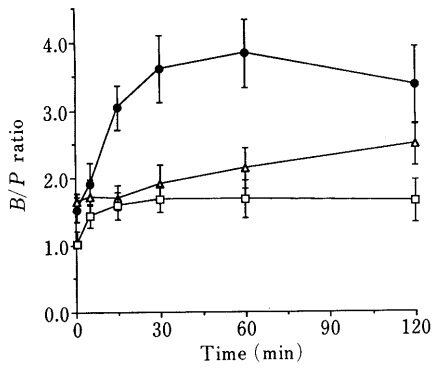

Fig. 1. Effect of Temperature on B/P Ratio of THP in Blood

Each medium was incubated with 0.319 μM of THP at various temperatures (37 °C; closed circle, 25 °C; open triangle, 0 °C; open square). Each point represents the mean ± S.D. N is 12 for 37 °C, and 4 for others.

Fig. 3. There was no relationship between the B/P ratios and the WBC counts ($r=0.300$, $p>0.05$). In addition, there was no relationship between the B/P ratios and the Ht values (data not shown).

Effect of Concentration on Distribution of THP in Blood Figure 4 shows that the B/P ratios at 1.59 μM (1000 ng/ml) were always greater than those at 0.319 μM (200 ng/ml) and there was an especially significant difference at 0.33 min after the addition of THP. The B/P ratio at 7.97 μM (5000 ng/ml) showed significantly smaller values than those of the two other groups at 15, 30 and 60 min after the addition of THP.

Stability of THP in Blood and Plasma The stability of THP in human blood or plasma at 37 °C is shown in Fig. 5A. The THP levels in plasma were similar to those in blood up to 15 min after the addition of THP. However, the THP in plasma decreased significantly compared with that in blood after 60 min, and at 240 min, the residual THP in plasma (29.6±2.69%) was significantly smaller than that in blood (90.2±5.71%, $p<0.001$). Regarding THP metabolism in blood at 37 °C, THP-OH was observed from 5 min after the addition of THP and increased time-dependently, but no THP-OH was detected in the plasma. Furthermore, there was no transformation of THP into ADR in either blood or plasma at 37 °C (Figs. 5B and C).

Figure 6 shows the fraction metabolized from THP to THP-OH in blood at various temperatures. The blood concentrations of THP-OH increased with longer incubation (Fig. 5C) and the THP-OH formed at 37 °C was 6.46±1.86% at 240 min after addition.

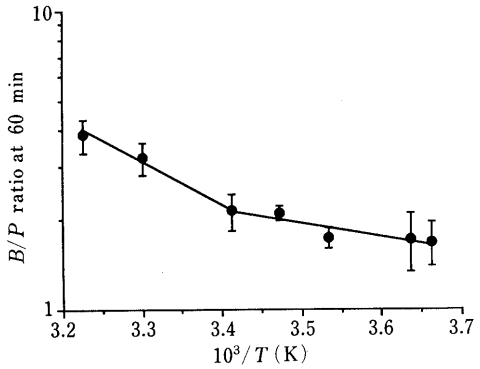

Fig. 2. An Arrhenius Plot for THP Uptake by Blood Cells

Each medium was incubated with 0.319 μM of THP for 60 min at various temperatures. Each point represents the mean±S.D. N is 12 for 37 °C for 4 for others.

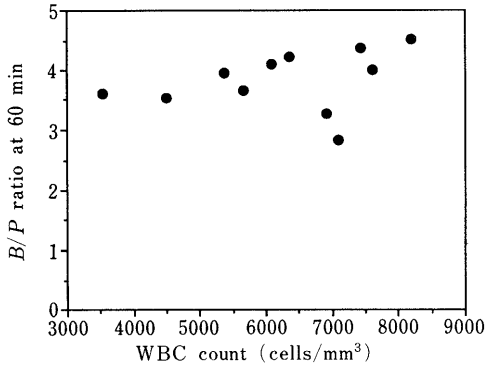

Fig. 3. Correlation between WBC Counts and the B/P Ratios of THP

Each medium was incubated with 0.319 μM of THP for 60 min at 37 °C. Each point represents the value of 11 subjects. $Y=0.000107X+3.16$. $r=0.300$ ($p>0.05$).

Discussion

In cancer chemotherapy it is important that antitumor agents are effectively taken up by the tumor region, *i.e.*, tumor cells.[6] Furthermore, the concentration of anthracycline derivatives in the cells is closely associated with the survival or growth rate of tumor cells. ADR had a steep dose–response effect both *in vitro* and *in vivo*.[7-11] However, in the case of ADR, no effects were noted below a certain threshold,[12] and at low concentrations, growth stimulation has even been observed. Above this threshold

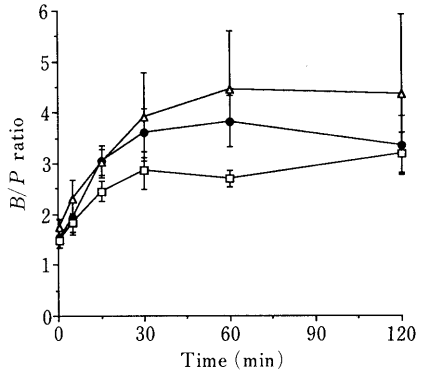

Fig. 4. Concentration Dependence of B/P Ratio of THP in Blood

Each medium was incubated with various concentrations of THP (0.319 μM; closed circle, 1.59 μM; open triangle, 7.97 μM; open square) at 37° C. Each point represents the mean±S.D. N is 12 for 0.319 μM, and 4 for others.

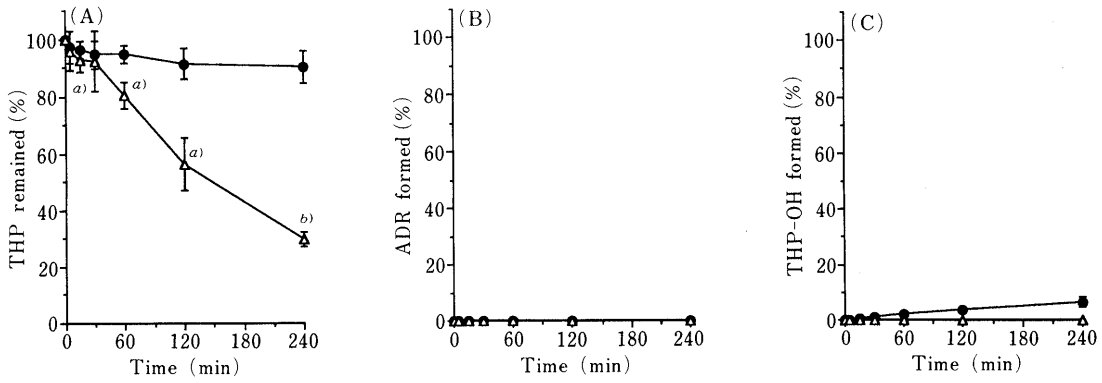

Fig. 5. Time Courses of Residual THP (A), and ADR (B) and THP-OH (C) Formed in Blood (Closed Circle) and Plasma (Open Triangle)

Each medium was incubated with 0.319 μM of THP at 37 °C. Each point represents the mean±S.D. N is 12 for the blood experiments and 3 for plasma experiments. *a*) $p<0.01$ and *b*) $p<0.001$, significantly different from blood value, respectively.

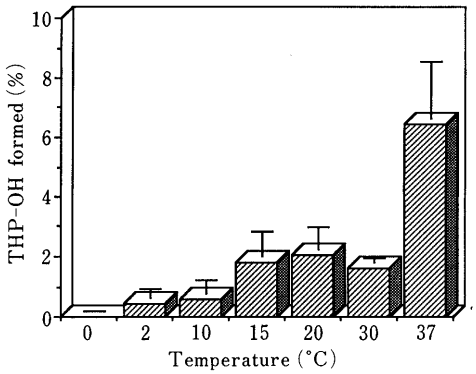

Fig. 6. Effect of Temperature on THP-OH Formation in Blood

Each medium was incubated with 0.319 μM of THP at 37 °C for 20 min. Each bar represents the mean ± S.D. N is 12 for 37 °C, and 4 for others.

level, a small increase in dose produces a considerable increase in both cytotoxicity and side effects.[13,14] Furthermore, it is reported that repeated administrations of ADR on successive days produced an accumulation of the drug at the cellular level, without concurrent accumulation in the plasma.[14] Thus, it is suspected that the normal cellular uptake of anthracyclines may possibly affect both cytotoxicity and side effects, because it is considered that those drugs are taken up by blood cells and the efflux is so slow that the tumor cells are subjected to low drug concentrations for an extended time. We previously reported that the uptake of THP by blood cells had large interindividual variations.[5] It is therefore suggested that the variation in the THP uptake by blood cells might be related to interindividual variations in the efficacy and the incidence of toxicity. In this report, in order to clarify the reason for the interindividual variations and the mechanisms of the uptake, we investigated the effect of temperature and drug concentration on the distribution of THP in blood, and the stability of THP in blood and plasma.

As shown in Fig. 1, the THP uptake by blood cells showed a temperature- and time-dependency. The THP B/P ratios increased with increasing incubation temperature and duration. In addition, there were decreases of the B/P ratio at 37 °C from 60 min after incubation. This probably accounts for why hemolysis progressed at 37 °C with consequent increases in THP concentration in the plasma fraction. In addition, when the incubation was conducted at temperatures above 10 °C, temperature- and time-dependent hemolysis was observed. With regard to the hemolytic activity of THP, Tone *et al.* reported that THP arose hemolysis at concentrations of 8×10^{-5} and 10^{-4} g/ml.[15] However, in this study, hemolysis was noted at THP concentrations of 200 ng/ml (2×10^{-7} g/ml). This result indicates that THP had hemolytic activity at lower concentrations than that reported by Tone *et al.*

To examine the dependency of THP uptake on temperatures at 0.319 μM in detail, an Arrhenius plot for THP uptake was plotted. As shown in Fig. 2, since the Arrhenius plot was biphasic, it is suspected that THP is taken up by blood cells by a carrier-mediated system.[16] This is supported by the results shown in Fig. 1 in which the B/P ratio at 37 °C reached a plateau at 60 min after drug addition, which indicates that the uptake of THP by blood cells had equilibrated. It was reported that both WBC and red blood cells (RBC) were involved in the uptake of THP in blood.[17-20] In order to confirm the participation of WBC or RBC counts in THP uptake, we examined the correlation between WBC counts or Ht values in the subjects and their B/P ratios. As shown in Fig. 3, the relationship between WBC counts and B/P ratios at 60 min and between Ht values and B/P ratios at 60 min was not significant. In addition, there were large interindividual variations (CV = 13.0%) in the B/P ratios, *i.e.* the uptake of THP by blood cells. It was therefore considered that other unknown factors, other than variations in blood cell counts such as WBC and RBC counts, were responsible for the variation in THP uptake by blood cells as shown in our previous report.[5]

Regarding concentration-dependency, since the B/P ratios at 1.59 μM were greater than those at 0.319 μM at all times, in particular, the B/P ratio at 0.33 min showed a significantly greater value ($p < 0.05$), this indicated the uptake of THP by blood cells had a concentration-dependent (Fig. 1). However, the B/P ratio at 7.97 μM showed significantly smaller values in comparison with two other concentrations at 15, 30 and 60 min, respectively. Since the THP added was in excess of the uptake capacity of the blood cells, consequently the proportion of THP in the plasma fraction was increased. Therefore it appeared that THP uptake by blood cells was concentration-dependent, and equilibrium was achieved at high concentrations.

When the stability of THP was investigated in human blood and plasma samples, the degradation rate of THP progressed with a lapse of time while the degradation rate at 37 °C was highest in comparison with that at other temperatures. Furthermore, the THP degradation rate in plasma showed much greater values compared to those in blood from 30 min after addition. These results indicated that THP was mainly degraded in the plasma fraction and it was protected from degradation by transportation into blood cells (Fig. 5A). Our results in which the half-life of THP in plasma was 2.29 ± 0.295 h approximately agreed with findings of Miller *et al.* (3.1 h).[17] On the other hand, it is known that an alcohol metabolite of anthracyclines has antitumor activity[21-24] and is related to the incidence of cardiotoxicity.[23] In this study, the formation of THP-OH from THP in blood showed temperature- and time-dependency, as shown in Figs. 5C and 6. However, THP-OH was not detected in the plasma experiment. These results indicated that THP was metabolized into THP-OH in blood cells *via* a metabolic pathway mediated by an enzyme. It was reported that the aldo-keto reductase in blood cells is related to metabolism of ADR into doxorubicinol.[14,15,25,26] It is considered that THP might be metabolized into THP-OH by the aldo-keto reductase in blood cells as with ADR. On the other hand, THP was not metabolized into ADR in either blood or plasma (Fig. 5B). These results indicated that THP was metabolized into THP-OH in blood cells by an enzyme and then the blood cells served to protect against the degradation of THP in blood.

In conclusion, the present study indicated that: (1) the distribution of THP in blood showed a marked temperature- and concentration-dependency, and THP uptake by blood cells reached equilibrium, (2) unknown factors besides WBC and RBC counts were related to the interindividual

variations in THP distribution in blood, (3) THP is metabolized into THP-OH in blood cells by an enzyme and then blood cells serve to protect against the degradation of THP in blood. In addition, these results suggested that when the monitoring of THP and active metabolites is carried out in patients, it is necessary to freeze both blood and plasma samples immediately after blood collection. In this study, we could not elucidate the reason for interindividual variations in THP uptake. In order to clarify the factors, additional studies are in progress by using unitary cells such as WBC and RBC.

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