Supplemental Information

1. Exclusion Criteria:

All patients were required to have left ventricular ejection fraction (LVEF) ≥ 50%; total bilirubin ≤ 2.0 mg/dL (34 μmol/L); serum creatinine ≤ 2.5 mg/dL (221 μmol/L); PT or PTT ≤ 1.5 times the institution’s upper normal limit (UNL); platelets ≥ 75,000/mm³; WBC ≥ 1500/mm³; and Hgb ≥ 10 g/dL. Exclusion criteria included serious co-morbid medical illnesses within the last 6 months; uncorrectable coagulopathy; or taking any of the following medications: Cyclosporine, Phenobarbital, Phenytoin, Streptozocin, or live vaccines. The study was conducted at two institutions to expand the population of patients and the recruitment of a wider variety of histologies.

2. Preparation of Liposomal Doxorubicin

The formulation process for Lyso-thermosensitive liposomal doxorubicin (LTLD) (ThermoDox®, Celsion Corp., Columbia, MD) used a pre-formed solution of 100 nm diameter liposomes, sodium carbonate solution, and 20 or 50mg vials of commercially available Doxorubicin Hydrochloride for Injection; USP. Aliquots (1.9ml) of the liposomes and sodium carbonate solution (1.2mL), to adjust the pH, were warmed to 35 ±1°C in a water bath. A solution of doxorubicin in sterile water for injection (5.88mg/ml) was equilibrated in the same water bath in a separate vial. Once at temperature, 1.6ml of doxorubicin was added to the pH adjusted liposomal solution, mixed gently, incubated at 35 ±1°C for 60 minutes and then allowed to cool to room temperature to give a mixture containing 2mg/mL doxorubicin and 40mg/mL lipids. During this process more than 90% of the doxorubicin was driven into the liposomes by the pH difference between the inside and the outside. A sample was compared against a color chart to determine the percent entrapment. For each subject, the assigned doxorubicin dose
was then diluted to 250 mL with 5% Dextrose in Water for Injection USP at room temperature.

3. Effect of heating on doxorubicin’s cytotoxicity

Heating doxorubicin to 90 °C for 12 min did not significantly change its cytotoxicity towards JC adenocarcinoma cells when compared with free doxorubicin. The percent of viable cells were measured at 1, 3, 6, and 24 hours after heated or “normal” doxorubicin treatment. Both heated and “normal” doxorubicin decreased the viability of the cells when incubation time was increased (P < 0.05, ANOVA), but the degree of viability was not significantly different between treatment groups (P = 0.8546, ANOVA). These results indicate that delivering doxorubicin during a characteristic RFA procedure will not negatively affect its potency.

4. Plasma and Urine Sampling

LTLD was administered as a nominal, 30-minute, 250 mL, intravenous infusion at assigned doses of 20, 30, 40, 50 and 60 mg/m². Actual doses were all >95% of assigned doses. On Day 1 before the start of the infusion (time = 0), blood and urine samples were obtained from all subjects to establish baseline values for the assays. Additionally, blood was drawn (4 mL, EDTA tubes) for determination of plasma doxorubicin and doxorubicinol concentrations at nominal times of 0.25, 0.5 (end of infusion), 0.75, 1, 1.25, 1.5, 2, 3, 4, 6, 24 and 48 hours after the start of the infusion, as well as on Day 4 (96 hours) and Day 8 (192 hours). Actual collection times were recorded and used in the calculation of pharmacokinetic parameters. Whole blood samples were maintained on ice, and centrifuged within 30 minutes of being drawn at 2000 x g for 10 minutes at 4°C. The resultant plasma was transferred to labeled polypropylene tubes and stored frozen at –70°C.

A urine sample was taken approximately 30 minutes before the start of infusion (baseline) and at the following intervals following the start of the infusion: 0-2, 2-4, 4-24
and 24-48 hours. A single urine sample was also collected on Days 4 and 8. Urine was collected in sterile containers, and stored under refrigerated conditions (2-8°C) until sampling. At the end of each interval, urine volume was recorded and two 1.5 mL aliquots were collected into labeled polypropylene tubes and stored frozen at -70°C. Plasma and urine samples were shipped frozen on dry ice for analysis.

5. Bioanalytical Methods

Plasma and urine was analyzed for total doxorubicin and the primary metabolite, doxorubicinol, employing validated LC/MS/MS methods. These methods were robust and free of interferences from concomitant, anti-hypersensitivity medications. Briefly, the method involved a methanol extraction of human (EDTA plasma samples Two standard curves were required to cover the observed plasma doxorubicin concentrations; the methods proved to be linear over the ranges of 0.5 ng/mL to 250 ng/mL and 0.2 µg/mL to 50.0 µg/mL, and the low level of quantification (LLOQ) for both doxorubicin and doxorubicinol in plasma was 0.5 ng/mL.

Analogously, another validated methanol extraction method and LC/MS/MS assay was used to quantitate doxorubicin and doxorubicinol in urine where the LLOQ for both analytes was 1.5 ng/mL.

6. Pharmacokinetic Methods

Plasma concentration-time data for total doxorubicin and the primary metabolite, doxorubicinol, were analyzed by non-compartment methods [17, 38]. Plasma and urine pharmacokinetic parameters were derived using standard software (WinNonlin, version 4.1.1 (2005) Pharsight Corporation, Mountain View, CA).

7. Plasma Pharmacokinetics

Some obvious outliers were identified and removed from the pharmacokinetic analysis based upon visual inspection of the plasma concentration – time curves for each subject. Since the LLOQ for total doxorubicin and doxorubicinol were 0.5 ng/mL, plasma
concentrations of these analytes measured before the start of the infusion (time 0) that were below LLOQ were set equal to zero; subsequent plasma concentrations below LLOQ were treated as missing data points.

The terminal exponential phase data points were determined by visual inspection of the plasma concentration-time profiles and the terminal exponential half-life (T½) and λz values were calculated by log-linear regression of terminal exponential phase data points. Maximum plasma concentration (Cmax) and time to Cmax (Tmax) were derived by WinNonlin algorithms and gave the same results as visual inspection. Areas under the plasma concentration-time curves were calculated using the linear trapezoidal rule up to the maximum plasma concentration, and thereafter using the logarithmic trapezoidal rule [39]. The area under the plasma concentration-time curve (AUC) was calculated to the last measurable plasma concentration (AUClast), where “last” refers to the time of the last measurable plasma concentration, and the remaining area was extrapolated to infinity by dividing the last measurable plasma concentration by the terminal exponential rate constant, λz. Summing these two segmented areas gave area under the plasma concentration-time profile from time zero to infinity (AUC0-∞). Since most of the AUC associated with each analyte occurred during the first 4 hours after the start of the infusion, the AUC over this period (AUC0-4) was calculated and expressed as a percent of AUC0-∞.

A full pharmacokinetic parameter profile of clearance (CL), volume of distribution at steady state (Vss), and mean residence time (MRT) [40] was determined for doxorubicin as the administered drug and for doxorubicinol.

Results of these calculations are presented in the Supplemental Tables 1 – 6 and the linearity of values for AUC0-∞ with dose is shown in Supplemental Figure 1.

8. Urine Pharmacokinetics

Urine was not available from 4 subjects, all at the 50 mg/m² dose. Urinary analytes for each individual entity were summed when there was a continuum of values during the collection intervals. For example, if the amount of an analyte was quantified over the 0-
2 hour period, but not for the 2-4 hour period, no further urine data was employed for calculation of renal clearance, even if all other urine amounts over the following intervals were available. Renal clearance (CLr) was calculated by taking the amount of each analyte excreted over the time interval, and dividing this by the corresponding plasma AUC. When plasma concentrations times did not match the urine collection times, WinNonlin was used to calculate AUC corresponding time values using the appropriate interpolation or extrapolation algorithm. Using the value for CLr, the amount of doxorubicin and doxorubicinol excreted in urine was extrapolated from time 0 to infinity (assuming linearity) using the product of renal clearance and AUC_{0-\infty}. These data and the ratio of parent drug to metabolite are shown in Supplemental Table 7 and the 4-day total urinary output per administered dose is shown in Supplemental Figure 2.

9. Pharmacokinetics during RadioFrequency Ablation (RFA)

The total time from the start of use of RFA (15 minutes after the start of the infusion) to the end of the RFA use (Overall RFA) was recorded for each subject. Because of the size of the tumors, multiple applications of radiofrequency were often required to ensure complete ablation. This resulted in periods when the RFA current was not switched on while the ablation needle was being re-positioned. The actual time during which the RFA current was switched on (Intermittent RFA) was also recorded for each subject. The summation of intermittent RFA and its percentage of overall RFA were calculated using standard software (Microsoft Excel, 2003).

Both the overall RFA and intermittent RFA times from each subject in the 50 mg/m² (maximum tolerated dose) cohort were used in WinNonlin to calculate the exposure (AUC) to doxorubicin during the RFA procedure and to relate this exposure to the subject’s overall exposure (AUC_{0-\infty}). These results are shown in Supplemental Table 8.

10. Linear Regression Analysis
The effects of dose and selected independent doxorubicin plasma pharmacokinetic parameters were co-analyzed with hematological pharmacodynamic data using linear regression [6]. Independent variables were: dose; maximum doxorubicin plasma concentration; and doxorubicin area under the plasma concentration – time curve from 0 to infinity; pharmacodynamic values were treated as dependent variables. Excluding data prior to dosing, these variables were: minimum/least absolute neutrophil count (least ANC); minimum/least white blood cell count (least WBC); and minimum/least platelet count (least platelets). In this context, “minimum/least” refers to the lowest measured hematological count after LTLD therapy for each subject, irrespective of pre-dose count or elapsed time post-treatment. The results for dose versus minimum/least absolute neutrophil count (least ANC) are shown in Supplemental Figure 3.

Results and Discussion

Supplemental Tables 1-5 show the doxorubicin pharmacokinetic parameters for each dose cohort. Following the end of the infusion, plasma doxorubicin levels undergo a complex polyexponential decay (based on visual inspection of plasma concentration – time curves), however, irrespective of administered dose, the majority (91.5%: range 79.96 – 94.87) of the subject’s overall exposure (AUC_{0-\infty}) to doxorubicin occurs in the first 4 hours after the start of the infusion. Therefore, the long terminal half-life (60.54 hr: range 48.67 – 79.92 hr) may have little clinical significance and drug elimination from the body is better characterized by the mean initial half-life (1.24 hr: range 0.96 – 1.68hr) or mean residence-time (3.57 hr: range 1.99 - 6.46hr). Supplemental Figure 1 shows that the mean values for AUC_{0-\infty} (and C_{max} – not shown) are linear with respect to dose suggesting that no saturation of metabolism occurs.

The total plasma clearance of doxorubicin is low (mean from all doses 1.2 L/hr /m²: range 0.75 – 3.6) and the drug distributes into a small volume at steady state (6.07 L/m²: with a broad range 2.1 – 21) or about 4 times the plasma volume. The low clearance of doxorubicin occurs despite the drug’s apparent rapid disappearance from
the circulation (MRT = 3.57 hr and 91.5% of the AUC is complete in 4 hours) because the plasma concentrations are high and the drug is distributed into a small volume while this elimination occurs.

The overall 5.3 hour $T_{max}$ of doxorubicinol (Supplemental Table 6) indicates it is not rapidly formed from doxorubicin and/or it is relatively slowly eliminated (clearance is approximately 24-fold slower than the parent drug). The low 4-hour value for AUC as % AUC$_{0-\infty}$ (3.85%) reflects the relatively low percentage of doxorubicin ultimately converted to doxorubicinol. The mean $T_{\frac{1}{2}}$ of 62.7 hours is slightly longer than that of doxorubicin and is consistent with published reports of Adriamycin metabolism [18]. The AUC ratio of doxorubicinol to unchanged parent drug has been reported [18] to be approximately 0.55 compared to 0.2 in this study, a difference that may reflect the extended plasma concentration – time profiles.

In Supplemental Table 7, the median renal clearance value for doxorubicin is shown as 0.36 L/hr, which corresponded to about 7.2 % of the dose excreted in urine from 0 to infinity. Renal clearance for doxorubicinol was calculated as 1.35 L/hr, corresponding to about 3.7 % of the dose excreted in urine from 0 to infinity. The total calculated urinary output (% excreted to infinity) of both doxorubicin and doxorubicinol for all doses was 10.8 % and this in broad agreement with the cumulative 4-day urinary excretion shown in Supplemental Figure 2. Published reports of adriamycin urinary excretion show less than 10% of the dose found in the urine, of which approximately $\frac{2}{3}$ was doxorubicin and $\frac{1}{3}$ was doxorubicinol [41].

The first 4 hours of exposure (AUC) to doxorubicin corresponding to majority of the total (AUC$_{0-\infty}$) exposure are the obvious window for performing RFA while the plasma concentrations are at their height. Supplemental Table 7 shows the overall and intermittent RFA times and their correspondence to the AUC$_{0-\infty}$ exposure and confirms that overall RFA times (93.9% of AUC$_{0-\infty}$) and intermittent RFA times (52.5% of AUC$_{0-\infty}$) are optimal during the 4 hour window.
The effect of dose was co-analyzed with hematological pharmacodynamic data using linear regression (Supplemental Figure 3). The figure shows that minimum/least absolute neutrophil count (and least white cell count – not shown) correlates well ($p = 0.025$) with administered dose of LTLD while least platelet count does not (not shown). Similarly, linear regression analysis (not shown) demonstrated doxorubicin $C_{\text{max}}$ and $\text{AUC}_{0-\infty}$ also correlated with both minimum/least absolute neutrophil count and minimum/least white blood cell count (least WBC); but not with minimum/least platelet count.
Reference List


Supplemental Figure 2

Urinary Excretion of Doxorubicin and Doxorubicinol Over 4 Days

Mean (± SD) % Injected Dose Excreted over 4 days

Dose (mg/m²)

- Urinary Doxorubicin
- Urinary Doxorubicinol
Supplemental Figure 3

Linear regression analysis of ANC vs Dose

Least ANC ($\times 10^9$/L)

Dose (mg/m$^2$)

$R^2 = 0.207$

$p = 0.0254$
Supplemental Figure 1
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