ABSTRACT:

Tumor-selective delivery of doxorubicin by a prostate-specific antigen (PSA)-targeted peptide conjugate prodrug of doxorubicin was demonstrated in a nude mouse xenograft model of human prostate cancer. The prodrug (referred to as doxorubicin conjugate) contains doxorubicin linked to a seven-amino acid peptide conjugate that was designed to increase delivery of doxorubicin to tumor sites through the hydrolytic properties of PSA, which produces in elevated amounts. PSA is a protease involved in the production of seminal fluid (Lilja et al., 1989). Systemic concentrations of PSA are elevated in the majority of prostate cancer patients. The PSA specificity of metabolism; LNCaP cells mediated rapid metabolism of the conjugate, while DuPRO-1 cells, which are deficient in PSA, were incapable of metabolism.

Targeted prodrugs can potentially increase delivery of active anticancer agents to tumors while minimizing systemic toxicity. One such targeting strategy uses the elevated levels of enzymes specific to tumor tissue to activate prodrugs to their corresponding active metabolites. The targeting strategy is exemplified by capecitabine, which is a prodrug used in the treatment of metastatic breast cancer. This inactive produg is metabolized to 5-fluorouracil by thymidine phosphorylase, an enzyme present in relatively high concentrations in tumor tissues (Ishikawa et al., 1998).

Prostate cancer that is confined to the organ is successfully treated with surgery or radiation; however, once the disease has spread beyond the organ the prognosis is relatively poor. The current therapy for metastatic prostate cancer is a combination of mitoxantrone and prednisone that provides response rates of 20 to 30% (Raghavan et al., 1997; Beedassy and Cardi, 1999). Although doxorubicin exhibits clinical activity in prostate cancer, its use is limited because of the systemic toxicities, primarily immunosuppression and cardiac toxicity (Raghavan et al., 1997). A delivery mechanism capable of increasing the delivery of the antitumor agent to the tumor while decreasing systemic exposure would increase the therapeutic index and response rates.

One method of targeting antitumor drugs to prostate tumors is to use prostate-specific antigen (PSA1), an enzyme that these tumors produce in elevated amounts. PSA is a protease involved in the hydrolytic processing of semenogelins, which is necessary for liquefaction of seminal fluid (Lilja et al., 1989). Systemic concentrations of PSA are elevated in the majority of prostate cancer patients. The PSA that is secreted into the blood is catalytically inactive because it circulates in a bound form, primarily to α1-antichymotrypsin and α2-macroglobulin (Lilja et al., 1991; Otto et al., 1998). Ideally, only PSA would be able to metabolize the prodrug to its active form, thereby producing high localized concentrations of the antitumor agent. Systemic exposure should be minimal, resulting only from the active drug that redistributes out of the cancerous tissue.

A prodrug that is a seven-amino acid peptide conjugate of doxorubicin has been optimized for maximal cleavage rate by PSA (Fig. 1). Cleavage maps indicate that PSA mediates hydrolysis of semenogelin-I at multiple sites (Denneade et al., 1997; Malm et al., 1997). Recent studies show that the glutamine-serine peptide bond (residues 349 and 350) of semenogelin-I is an important cleavage site, and this site is contained in the peptide portion of the doxorubicin conjugate (Fig. 1). The doxorubicin conjugate exhibits superior activity in vitro versus LNCaP cells, a human prostate tumor cell line that produces PSA (DeFeo-Jones et al., 2000). The current studies demonstrated that the doxorubicin conjugate provided selective tissue distribution of doxorubicin in an in vivo human xenograft murine model of prostate...
was calculated as \( C_{\text{20}} \text{ml/min} \). The extraction ratio, a measure of the efficiency of hepatic removal, doxorubicin) in 0.3% glucose/Krebs-Henseleit (pH 7.4) buffer at a flow rate of 1.6 ml/min. The retention times of doxorubicin, increase from 20 – 44% from 0 – 30 min, held at 44% acetonitrile from 30 – 33 min. The hepatic extraction of doxorubicin conjugate was calculated as \( \text{Dose}_{\text{i.v.}} / \text{AUC} \), while the terminal half-life was estimated from the slope of the log-linear portion of the plasma concentration-time profile. The distribution volume \( (V_{\text{ss}}) \) was calculated as \( [\text{Dose}_{\text{i.v.}} - \text{AUMC}] / [\text{AUC}^2] \), where AUMC is the area under the first moment curve.

To assess the selectivity of PSA-mediated hydrolysis by tumor cells, the metabolism of the doxorubicin conjugate was examined in a prostate tumor cell line that produces PSA (LNCaP) and one that does not (DuPRO-1). The time course of concentrations of parent drug (initial concentration: 0.6 \( \mu \text{M} \)) and its active metabolites leucine-doxorubicin and doxorubicin was determined in the cell suspension (ca. 0.5 \( \times 10^6 \text{cells/ml} \)) at 37°C.

Tissue Distribution Study. Male nude mice (Taconic Farms, Germantown, NY) weighing approximately 25 g were used for the pharmacokinetic studies of the doxorubicin conjugate and its active metabolites, leucine-doxorubicin and doxorubicin. In a tissue distribution study, the concentrations of parent drug, leucine-doxorubicin, and doxorubicin in tumor and select tissues were determined in PSA-producing tumor-bearing nude mice. Tumor cells (LNCaP) were implanted subcutaneously and allowed to grow for about 4 weeks until the tumor weight was about 1 g (Horoszewicz et al., 1983). After i.p. administration of an equimolar dose of doxorubicin conjugate (20 mg/kg) and doxorubicin (8 mg/kg), the tumor, heart, liver, kidney, and brain were excised at 0.25, 0.5, 1, 2, 4, and 24 h post dose (\( n = 3 \) per time point) and frozen in liquid nitrogen until assayed. Blood was drawn by cardiac puncture and the resultant plasma frozen on dry ice.

Fraction Metabolized to Doxorubicin. To assess the pharmacokinetics of doxorubicin conjugate and the fractional conversion to doxorubicin, nude mice, rats, dogs, and monkeys received an i.v. dose of doxorubicin conjugate or a dose of doxorubicin. Plasma samples were obtained periodically after 4 h and stored frozen at −70°C until analyzed.

Isolated Perfused Rat Liver. The hepatic extraction of doxorubicin conjugate and its metabolites, leucine-doxorubicin and doxorubicin, was determined in duplicate using the single-pass isolated perfused rat liver technique (Pang, 1984). These compounds were perfused via the portal vein at concentrations of 0.1 (doxorubicin conjugate) or 1 \( \mu \text{M} \) (leucine-doxorubicin and doxorubicin) in 0.3% glucose/Krebs-Henseleit (pH 7.4) buffer at a flow rate of 20 ml/min. The extraction ratio, a measure of the efficiency of hepatic removal, was calculated as \( [C_{\text{in}} - C_{\text{out}}] / C_{\text{in}} \times 100\% \), where \( C_{\text{in}} \) and \( C_{\text{out}} \) are the influent and effluent concentrations in the perfusion media. The \( C_{\text{out}} \) was measured every 10 min over an approximately 60-min period when the extraction ratio was constant (minimum of six measurements). In a separate experiment, livers were perfused with \( ^{14} \text{C} \)leucine-doxorubicin, and the composition of radioactivity excreted in bile was profiled by LC with radiometric detection using an Agilent Mac-Mod Zorbax RX-C18 (4.6 × 250-mm) column (Agilent, Chadds Ford, PA). Elution was performed with a gradient of acetonitrile in aqueous 0.3% trifluoroacetic acid, 0.1% triethylamine (pH 3, linear increase from 20–44% from 0–30 min, held at 44% acetonitrile from 30–33 min) at a flow rate of 1.6 ml/min. The retention times of doxorubicin, leucine-doxorubicin, and doxorubicin conjugate were 17, 20, and 26 min under these conditions.

Uptake of Doxorubicin Conjugate and Its Active Metabolites into Human Prostate Tumor Cell Lines. The LNCaP and DuPRO-1 cell culture models were used to assess the ability of doxorubicin conjugate and its active metabolites to distribute into tumor cells. Radiolabeled doxorubicin conjugate, leucine-doxorubicin, and doxorubicin were incubated (triplicate determinations) with two prostate tumor cell lines, LNCaP (PSA-producing) and DuPRO-1 (non-PSA-producing). The concentration of total radioactivity in the tumor cells and incubation medium was used to calculate an equilibrium cell-to-medium distribution ratio. A separate study examined the effect of verapamil (10 \( \mu \text{M} \)), an inhibitor of P-glycoprotein-mediated transport, on the accumulation of doxorubicin in LNCaP and DuPRO-1 tumor cells.

Pharmacokinetic Analysis. The plasma clearance of the doxorubicin conjugate was calculated as \( \text{Dose}_{\text{i.v.}} / \text{AUC} \), while the terminal half-life was estimated from the slope of the log-linear portion of the plasma concentration-time profile. The distribution volume \( (V_{\text{ss}}) \) was calculated as \( [\text{Dose}_{\text{i.v.}} - \text{AUMC}] / [\text{AUC}^2] \), where AUMC is the area under the first moment curve.

The fraction of the i.v. dose of doxorubicin conjugate metabolized to systemically available doxorubicin (fm) was calculated by comparing the AUC of doxorubicin after administration of doxorubicin conjugate (AUC\( ^{\text{C5}} \)) and doxorubicin (AUC\( ^{\text{M}} \)), where M is the molar dose of doxorubicin conjugate and \( \text{fm} = \frac{\text{AUC}^{\text{C5}} \times \text{M}}{\text{AUC}^{\text{M}}} \).

Assays for Doxorubicin Conjugate, Leucine-Doxorubicin, and Doxorubicin. Plasma, urine, and bile. Before chromatographic analysis, the biological fluids underwent either solid-phase extraction with C2 (urine) or C8 (plasma) cartridges or liquid-liquid extraction with methylene chloride (bile) to remove endogenous chromatographic interferences. Concentrations of doxorubicin conjugate, leucine-doxorubicin, and doxorubicin in the plasma, urine, and bile were determined by LC with fluorescence detection and the chromatographic conditions used for analysis of bile. The excitation wavelength was 480 nm and the emission wavelength was 560 nm.

Tissues. Tissues were homogenized with a motor-driven Teflon and glass tissue homogenizer in 1 volume of 10 mM ammonium acetate (pH 5.7) and proteins precipitated by the addition of 2 volumes of acetonitrile. Following centrifugation, the concentrations of doxorubicin conjugate, leucine-doxorubicin, and doxorubicin were determined by an LC-tandem mass spectrometry method. Briefly, analyses were performed with a Sciex (Concord, Ontario, Canada) model API3+ triple quadrupole mass spectrometer interfaced via an electrospray interface to a liquid chromatograph. Separation was achieved with an Agilent Mac-Mod Zorbax XDB-C8 column (4.6-mm i.d. × 7.5 cm, 5 \( \mu \text{m} \)). The isotropic mobile phase consisted of 45% solvent A (90% acetonitrile, 10% mM ammonium acetate, 0.1% w/v formic acid) and 55% solvent B (10% methanol, 90% 10 mM ammonium acetate, 0.1% w/v formic acid) at a flow rate of 0.8 ml/min. Doxorubicin conjugate, leucine-doxorubicin, and doxorubicin were detected in the positive ion mode by selected reaction monitoring using the mass transitions \( m/z \) 1419 to 1419, \( m/z \) 657 to 243, and \( m/z \) 544 to 361, respectively. The limits of quantification for the assay were 12.5 ng/ml for doxorubicin conjugate in plasma and 25 ng/g for doxorubicin conjugate in tissues; 6.25 ng/ml for leucine-doxorubicin in plasma and 12.5 ng/g for leucine-doxorubicin in tissues; and 6.25 ng/ml for doxorubicin in plasma and 12.5 ng/g for doxorubicin in tissues.

Results

Metabolism of the Doxorubicin Conjugate by LNCaP and DuPRO-1 Human Prostate Tumor Cell Lines. To assess the involvement of PSA in the conversion of the doxorubicin conjugate, the metabolism of doxorubicin conjugate was examined in cell culture with PSA-producing and non-PSA-producing human prostate tumor cell lines. Within 60 min, the LNCaP cells, which produce PSA, mediated essentially complete hydrolysis of doxorubicin conjugate (Fig. 2). The appearance of leucine-doxorubicin accounted substantially for the disappearance of doxorubicin conjugate. Over a 90-min incubation period, only a minor amount of doxorubicin was formed. In a separate experiment, after a 24-h incubation of the doxorubicin
conjugate with LNCaP cells, only doxorubicin was detected. Following incubation with DuPRO-1 cells, which are PSA-deficient, the metabolism of doxorubicin conjugate was minor, and there was relatively little formation of leucine-doxorubicin and doxorubicin.

Tissue Distribution in Tumor-Bearing Nude Mice. Following i.p. administration of doxorubicin conjugate to tumor-bearing mice, doxorubicin conjugate concentrations declined rapidly; by 4 h, concentrations in the tumor were below the quantification limit of the assay in all tissues. Leucine-doxorubicin was formed rapidly from doxorubicin conjugate as the peak concentration of 5.40 μM in tumor was observed at 1 h. Although initially lower in the doxorubicin conjugate-treated group, by 4 and 24 h, the concentrations of doxorubicin in tumor were 3 times those achieved after administration of an equimolar dose of doxorubicin itself (Table 1). The peak concentration in heart was less than one-fifth and the AUC was less than half that in mice receiving an equimolar dose of doxorubicin. As compared with tumor tissue, concentrations of leucine-doxorubicin in heart tissue were relatively low and contributed to total exposure only to a minor extent. In liver, as in the heart, the relative doxorubicin concentrations in the animals that received doxorubicin conjugate were lower than in comparable animals that received doxorubicin. In kidney and brain, the two treatments resulted in similar exposure to doxorubicin.

Pharmacokinetics and Fraction of the Dose Metabolized to Doxorubicin. Following i.v. administration to mice, rats, dogs, and monkeys, overall pharmacokinetic behavior was similar: plasma clearance of doxorubicin conjugate was moderate relative to the corresponding hepatic blood flow, distribution volume was less than or equal to total body water, and terminal half-life was less than 1 h (Table 2). The fraction of the i.v. dose metabolized to doxorubicin, which represents non-PSA-mediated conversion of the conjugate, was similar in these four species; about one-third of the dose (range: 28 – 41%) was converted to doxorubicin. In the tumor-bearing nude mice, the fraction of the dose converted to doxorubicin was 87%, which was greater than that measured in non-tumor-bearing nude mice.

In the isolated perfused rat liver, the mean hepatic extraction ratios for doxorubicin conjugate, leucine-doxorubicin, and doxorubicin were 11, 82, and 27%, respectively. Profiling of bile showed that the high extraction ratio for leucine-doxorubicin was due to biliary excretion of unchanged leucine-doxorubicin; radioactivity in bile consisted predominantly (94%) of leucine-doxorubicin.

Uptake of Doxorubicin Conjugate and Its Active Metabolites into LNCaP Tumor Cells. The active metabolites of doxorubicin conjugate, namely leucine-doxorubicin and doxorubicin, distributed more readily into tumor cell than did the parent compound (Table 3). As measured by the cell-to-medium ratio, leucine-doxorubicin and
doxorubicin distributed 5 and 50 times better than doxorubicin conjugate into LNCaP tumor cells.

**Effect of Verapamil on the in Vitro Uptake into Human Prostate Tumor Cells.** The potential for active transport-mediated efflux of doxorubicin from prostate tumor cells was examined using the LNCaP and DuPRO-1 cell lines. Verapamil, a potent inhibitor of the P-glycoprotein efflux transporter, had no effect on the cellular accumulation of doxorubicin, which suggested that in these cell lines this transporter is not involved in the uptake of doxorubicin.

**Discussion**

In a nude mouse xenograft model of human prostate cancer, administration of the doxorubicin conjugate more than doubled the tumor exposure to its active metabolite, doxorubicin, as compared with that achieved with an equimolar dose of doxorubicin itself (Table 1). Peak concentrations of leucine-doxorubicin, another active metabolite of doxorubicin conjugate, were relatively high, but the metabolite was eliminated more rapidly than doxorubicin. The increased tumor tissue concentrations were consistent with the in vitro assessment of cell uptake, which showed that these active metabolites of doxorubicin conjugate distributed avidly into tumor cells (Table 3). Against the LNCaP human prostate tumor cell lines, the in vitro cytotoxic potency of leucine-doxorubicin is only about one-ninth that of doxorubicin. Therefore, adjusted for its lower potency, leucine-doxorubicin contributed only an additional 16% (expressed as doxorubicin equivalents) to in vivo tumor exposure. As such, the antitumor effect of the doxorubicin conjugate was attributed primarily to doxorubicin. The increase in tumor exposure was pharmacologically meaningful; in murine efficacy studies in vivo doses of 20 mg/kg (weekly × 5) inhibited LNCaP tumor growth >80% (DeFeo-Jones et al., 2000). In contrast to the higher concentrations in tumor, the cardiac tissue of the doxorubicin conjugate-treated mice contained substantially lower quantities of doxorubicin. In heart tissue, the AUC was less than half and the \( C_{\text{max}} \) was less than one-fifth of that in the mice that received doxorubicin itself. Peak concentration in heart tissue appears to be an important determinant of doxorubicin-induced cardiac toxicity in patients since continuous infusion regimens are associated with a decreased incidence of cardiac toxicity when compared with rapid administration (Singal and Iliskovic, 1998). If lower cardiac concentrations are achieved in patients, this implies that with regard to toxicity, doxorubicin conjugate may have the potential for an improved safety margin as compared with doxorubicin itself. Exposure to doxorubicin also was lower in other key tissues with the exception of kidney. However, the kidneys were not perfused before analysis, and it is unclear what fraction of doxorubicin is attributable to contamination by residual urine. In preliminary studies of doxorubicin conjugate in rats and dogs, doxorubicin in urine accounted for 10% and 4% of dose, respectively (unpublished data). Administration of the doxorubicin conjugate has not been associated with kidney or brain pathology in mice at doses up to 35 mg/kg/week (DeFeo-Jones et al., 2000).

In an in vitro model of human prostate cancer, the metabolism of doxorubicin conjugate was PSA-dependent. Over a 90-min incubation, LNCaP cells mediated extensive metabolism, while DuPRO-1 cells, which are PSA-deficient, were incapable of metabolizing the doxorubicin conjugate. The disappearance of parent drug was accounted for substantially by the appearance of leucine-doxorubicin (Fig. 2). Serine-leucine-doxorubicin is the expected immediate product of PSA-mediated hydrolysis, and the rapid appearance of leucine-doxorubicin indicates that the intermediate underwent rapid metabol-
The subsequent conversion of leucine-doxorubicin to the more active species, doxorubicin, was a relatively slower process. This was consistent with the in vivo observation in tumor tissue of rapid formation of leucine-doxorubicin and relatively prolonged time course of doxorubicin (Fig. 3). Once formed, both leucine-doxorubicin and doxorubicin would be expected to distribute avidly into tumor tissue (Table 3). Also, the intact conjugate may distribute into tumor cells and become susceptible to hydrolysis mediated by membrane-bound PSA.

In some types of cancer, anthracycline chemotherapy is ineffective because of rapid P-glycoprotein-mediated efflux of drugs from tumor cells (Booser and Hortobagyi, 1994). Doxorubicin is a well known substrate of P-glycoprotein; however, this transporter is not expressed in LNCaP prostate tumor cells and is not considered the primary reason for the poor clinical efficacy of doxorubicin in prostate disease (Fig. 4; Theyer et al., 1993). Rather sufficient concentrations of doxorubicin cannot be achieved in prostate tumors without unacceptable toxicity in other tissues. A therapeutic modality, such as a prodrug, that provides selective delivery of doxorubicin to the prostate tumor may overcome, at least partially, this limitation of doxorubicin. The current studies show that this PSA-targeted prodrug selectively increased tumor delivery of doxorubicin and simultaneously decreased undesired distribution to the heart.

While doxorubicin conjugate provided selective delivery of doxorubicin to tumor tissue, there was substantial non-PSA-mediated formation of doxorubicin in laboratory animals. Following i.v. administration to mice, rats, dogs, and monkeys, about one-third of an i.v. dose was metabolized to doxorubicin (Table 2). In tumor-bearing mice, the fraction of the dose converted to doxorubicin was more than twice that in equivalent mice that lacked tumors, a consequence of formation in the tumor by PSA-specific hydrolysis. The tumor (1 g) represents a larger proportion of body weight in mice than it does in patients. Consequently, less PSA-specific conversion is expected clinically, and the systemic exposure to doxorubicin in tumor-bearing mice may overestimate that in patients. Although normal prostate tissue produces low basal levels of PSA, the serum PSA is catalytically inactive because it circulates bound to α1-antichymotrypsin and α2-macroglobulin (Lilja et al., 1991). Non-PSA-specific metabolism occurs by as yet uncharacterized proteases. The incomplete conversion of doxorubicin conjugate to doxorubicin appeared to be due, at least in rats, to rapid elimination of the intermediate metabolite, leucine-doxorubicin. In a single pass through the liver about 82% of the leucine-doxorubicin was excreted unchanged into bile. The hepatic extraction of the doxorubicin conjugate and doxorubicin was much less extensive. As would be expected from the partial conversion of the doxorubicin conjugate to doxorubicin, the acute doselimiting toxicities of the doxorubicin conjugate in mice are the same as those resulting from the administration of doxorubicin itself (DeFeo-Jones et al., 2000).

Despite the encouraging tissue distribution results in mice, it remains to be determined whether the increased delivery to prostate tumor tissue and accompanying efficacy can be achieved in patients. In nude mouse xenograft models, the tumor is implanted and grows subcutaneously and may respond differently to systemically administered drugs as compared with an anatomically normal tumor. According to the targeted drug delivery model described by Rowland and McLachlan (1996), key determinants of therapeutic gain are high systemic clearance of the active drug and low blood flow to the target organ. This provides the maximum benefit because the active drug (i.e., doxorubicin) formed within the target tissue eventually redistributes and enters the systemic circulation. Rapid clearance of the active drug from the systemic circulation minimizes toxicity from the specifically formed drug leaving the target organ. In the case of doxorubicin conjugate, systemic exposure to doxorubicin from the PSA-mediated conversion in the tumor would be a function of the amount formed and its clearance. The reported values for doxorubicin and leucine-doxorubicin clearance in cancer patients are in the range of 24 to 80 l/h (Speth et al., 1988; Canal et al., 1996), which are moderate to high relative to hepatic blood flow. Blood flow to prostate tissue is ca. 0.72 ml/min (assuming 25 g organ weight); therefore, doxorubicin clearance greatly exceeds organ blood flow (Vaupel et al., 1989). Since the doxorubicin conjugate is a prodrug, the extent of nonspecific doxorubicin formation also will influence its therapeutic gain relative to doxorubicin itself. In four preclinical species, about one-fourth to one-third of the dose of doxorubicin conjugate was converted non-specifically to doxorubicin, and the extent to which this occurs in patients will limit the maximum dose that can be administered. Ongoing Phase 1 clinical studies indicate that doxorubicin conjugate is well tolerated in patients, and preliminary data indicates its effects are attributable to doxorubicin (DiPaola et al., 2000).

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References


