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Design of an IV Formulation of an Unstable Prodrug Candidate for Prostate Cancer Treatment: Solution Chemistry of N-(glutaryl-hyp-ala-ser-cyclohexylglycyl-gln-ser-leu)-doxorubicin

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ABSTRACT The chemical degradation of N-(glutaryl-hyp-ala-ser-cyclohexylglycyl-gln-ser-leu)-doxorubicin (henceforth referred to as doxorubicin peptide conjugate **1**) was studied in buffered aqueous solution. The pH-rate profile of degradation shows that the doxorubicin conjugate is most stable between pH 5 and 6. The dependence of log k_{obsd} on pH in acidic medium is characteristic of specific acid-catalysis of the sugar hemiaminal of **1** (as in the case of doxorubicin). Isolation of degradates and structural determination shows that the degradation at lower pH values yields the water-insoluble aglycone doxorubicinone, supporting the mechanism of acid-catalyzed loss of the amino sugar. At pH higher than 5, a more complicated degradation pattern is observed, including the loss of the amino sugar and the aromatization of the saturated ring to give 7,8-dehydro-9,10-desacetyldoxorubicinone as one of the major products. Around the pH of maximum stability in solution, the rate of degradation of **1** is significantly greater than that for doxorubicin, which rules out the formulation of a room temperature solution product with a sufficiently long shelflife for market use. Design of a stable lyophilized formulation for sterile reconstitution based on the physicochemical properties of **1** is described.

KEYWORDS Doxorubicin peptide conjugate, Prodrug, Prostate cancer, Stability, Kinetics, Degradation, Preformulation, Lyophilization, IV formulation

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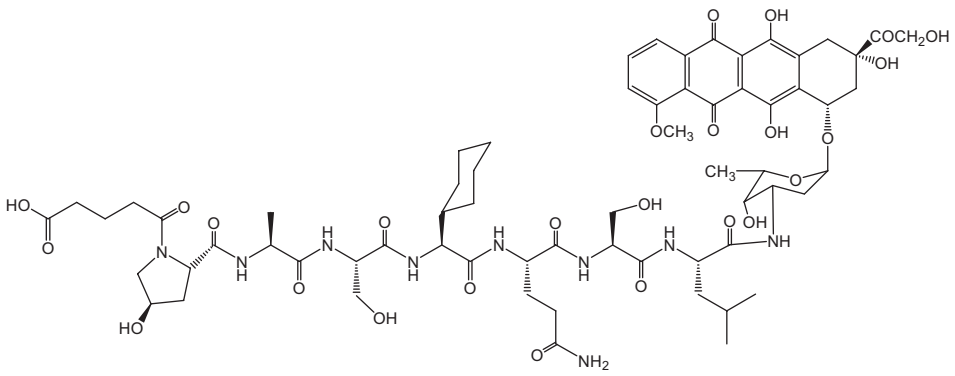
INTRODUCTION

Prostate cancer chemotherapy has traditionally shown limited effectiveness in prolonging life for patients with androgen-refractory cancer. (Husain et al., 1996) The standard combination of mitoxantrone and prednisone shows a relatively low (20–30%) response rate (Beedassy & Cardi, 1990). The use of the



Doxorubicin Peptide Conjugate **1**

FIGURE 1 **N-(glutaryl-hyp-ala-ser-cyclohexylglycyl-gln-ser-leu)-doxorubicin.**

anthracycline antibiotic doxorubicin (Arcamme, 1978) in treating prostate cancer is limited by its systemic toxicities, especially cardiotoxicity and immunosuppression. Prodrugs that reduce the systemic exposure of doxorubicin and provide altered tissue distribution may provide a safety advantage (D'Souza & Topp, 2004). Indeed, for doxorubicin, a stealth liposome formulation has shown to be associated with decreased cardiotoxicity, but the development of stomatitis and severe mucocutaneous toxicities limited further use in prostate cancer (Hubert et al., 2000). Doxorubicin peptide conjugate **1** (Fig. 1) is a cytotoxic agent proposed for the treatment of refractory prostate cancer by intravenous (IV) administration. This peptide conjugate has been designed to be a prostate-targeted prodrug that is nontoxic until activated by proteolytic cleavage to doxorubicin by locally expressed prostate-specific antigen (PSA). The design and synthesis of PSA-selective peptide conjugates of doxorubicin was reported recently (Garsky et al., 2001). The biological rationale for the selection of PSA-cleavable peptide linked to doxorubicin was also described recently (De Feo-Jones et al., 2000).

Investigation of the physicochemical properties of a drug candidate is an essential part of the development of a dosage form, because one needs insight into the reactivity of the molecule and environmental factors affecting its stability. The two key preformulation activities necessary to define an IV formulation are 1) quantitative determinations of solubility across the desired pH range for the most stable form of the drug molecule and 2) construction of a pH-rate profile across the pH range of interest (pH 2–8). The preparation and formulation of the peptide conjugate poses several challenges including the decreased chemical stability of **1** relative to doxorubicin. This compound also forms micelle-like aggregates in solution, further complicating the parenteral formulation. (Karki & Ostovic, 2004). Herein we describe the solution chemistry, degradation mechanism, and excipient impact on the stability of **1**. The formulation of a stable lyophilized composition of **1** for safety and efficacy study in prostate cancer is also described.

EXPERIMENTAL

Materials

Laboratory samples of N-(glutaryl-hyp-ala-ser-cyclohexylglycyl-gln-ser-leu)-doxorubicin were obtained from the medicinal chemistry group in Merck Research Laboratories at West Point, PA (USA). All samples were greater than 99% pure as judged by reversed phase HPLC and used without further purification. Doxorubicin was obtained from Sigma and used as received. All solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA) and were HPLC grade or equivalent. Reagents were obtained from Fisher and were certified as ACS grade.

Solubility

Conventional equilibration of excess solid in buffered aqueous media was used, followed by supernatant analysis by HPLC. The pH of the supernatant was also measured. The excess solids in equilibrium were analyzed by

optical microscopy to ascertain crystallinity: **1** shows weak diffraction peaks in powder XRD, and equilibration in buffers did not lead to improvement in this pattern. The material is microcrystalline (birefringent under optical microscope but does not show crystalline pattern by XRPD) both as received and through physicochemical evaluation. A crystalline sodium salt has been described (Karki et al., 2001).

Physical Stability of Solutions

Quasi-Elastic laser Light Scattering (QELS) experiments were performed with a BI 200 SM Goniometer (Brookhaven Instrument) equipped with a 10 mW HeNe laser (Melles Griot) as previously described (Karki & Ostovic, 2004; Karki et al., 2001). The hydrodynamic diameter obtained from QELS is considered an apparent particle size (Feng & Schelly, 1995; Phillies, 1990).

Solution Kinetics

Samples of **1** were prepared in various buffers and pH and then filtered through a 0.22-μm disk filter. The filtrate was filled in about 2-mL portions into glass ampules and flame sealed. Samples were stored in constant temperature ovens at 25, 40, 60, and 80°C (±0.5°C). Samples were removed from storage at recorded time intervals and allowed to reach room temperature. The samples were quantitatively diluted in deionized water and assayed for intact doxorubicin peptide conjugate and degradate levels by reversed phase HPLC. HPLC analyses were performed on a Spectra System HPLC equipped with Spectra System UV 2000 detector along with Spectra System P4000 pump and AS 3000 cooled autosample holder. The column used was a Waters Symmetry C18 (250 mm × 4.6 mm, 5-μm particle size). The UV detection wavelength was set at 250 nm, and the column was kept at ambient temperature.

Degradate Isolation

The acid-catalyzed degradate was isolated by treating the doxorubicin peptide conjugate with 0.1N HCl (pH 2.2). The aqueous solution was heated to 70°C for 30 min at which time precipitates were observed. The reaction mixture was cooled to room temperature, and the precipitate was filtered and washed with excess water. The isolated solid was vacuum dried overnight and found to be greater than 98% pure by HPLC. The structure was identified as doxorubicinone by ^{1}H NMR and mass spectrometry. The base-catalyzed degradate was isolated from high pH solutions using a preparative HPLC system (Rainin) through a Vydac Protein and Peptide C18 column (5 × 25 cm). The samples containing the degradate were combined and the solvent evaporated. The resulting solid was analyzed by ^{1}H NMR and mass spectrometry and found to correspond to 7,8-dehydro-9,10 desacetyldoxorubicinone.

Lyophilization

Lyophilization of solutions containing doxorubicin peptide conjugate and sugars was done on an FTS System (Dura-Dry™) lyophilizer with a stoppering tray. The following drying cycle was used for the samples studied:

1. Cool tray to –45°C at 1.0°C/min and hold for 120 min.
2. Raise to primary drying temperature of –25°C at 0.2°C/min, vacuum at 25 mT, and hold for 1000 min.
3. Raise at 0.2°C/min to secondary drying temperature of 15°C and hold for 600 min with vacuum at 25 mT.
4. Raise to 40°C at 1°C/min and hold for 180 min.
5. Lower to 20°C at 0.2°C/min and hold for 1000 min at 25 mT.

The samples were stoppered in the freeze dryer under vacuum and removed for analysis.

Thermal Analysis

DSC analysis was performed on an M-DSC system (TA Instruments). The solid lyophilized samples were removed from the stoppered vials in a glove box under nitrogen and then the samples sealed in aluminium pans in the glove box. The DSC run was modulated (±0.800°C every 60 sec), and the temperature was increased at 5°C/min to 150°C. Typically, the run was started after cooling the sample at 0°C for 2 min.

RESULTS AND DISCUSSION

Solubility

Although doxorubicin HCl is a water-soluble compound, the doxorubicin peptide conjugate **1** in its free

acid state is sparingly soluble in water. The solubility of **1** free acid was determined at room temperature to be 13.8 mg/mL, and the equilibrium pH of the resulting solution was 3.40. This value is lower than the observed solubility of greater than 100 mg/mL for doxorubicin HCl. The glutaryl moiety of **1** was added to the peptide terminus to increase the aqueous solubility of the prodrug. It was found through the course of medicinal chemistry optimization that in the absence of an ionizable functional group, the solubility of doxorubicin peptide conjugate would indeed be too low to support an IV product, in part because the high doses required for efficacy and safety assessment. Solubility enhancement was necessary to give the large IV doses necessary in toxicological studies. Thus, solubility issues were taken into consideration while designing the peptide sequence attached to doxorubicin (Garsky et al., 2001). It was anticipated that solubility of the doxorubicin peptide conjugate would be greatly enhanced as the pendant carboxyl group ionizes in the neutral pH range.

The equilibrium solubility values of free acid **1** at different pH values are summarized in Table 1. The solubility is greater than 156 mg/mL at pH 5.6 and higher; hence, high solubility was achieved through the compound's design. The pK_a for the doxorubicin conjugate (glutaric acid –COOH function) was determined titrimetrically to be 4.8 at room temperature (23°C). The value is in good agreement with the known pK_a of butyric acid (4.81 at 20°C). Solution of **1** is deep red, and a significant amount of self-association is occurring based on NMR. (Karki & Ostovic, 2004). The impact on physical properties such as solution viscosity (gelling) and other handling parameters is not significant in the pH range of 5.2 to above pH 8 (i.e., the ionized form of the compound is highly soluble and particles remain very small). Laser light scattering measurements of concentrated (40 mg/mL) solutions of **1** at pH 5.6 showed an apparent particle size (measured as the hydrodynamic ratio D_h) at 25°C of 2.7–3.2 nm; this size range was stable over the course of a week. Larger aggregates begin to form in 1 month at 25°C (D_h of 6.0 nm), and submicron precipitate forms on standing of the same solution in 1 month at 40°C (no precipitate was visible at 2 weeks). The precipitate is the insoluble doxorubicinone aglycone (vide infra). At refrigerated temperatures, the solution shows evidence of aggregation. At 5°C in 1 month, particles in the range of 7–8 microns were detected by light scattering and optical microscopy. The conclusion from solubility measurements and other physical stability assessment is that a solution formulation of **1** for long-term storage is not likely to be viable. Lyophilization will be explored as the alternative to improve storage stability. The development of the prelyophilization solution is predicated on finding the pH of maximum stability.

TABLE 1 **Equilibrium Solubility Measurements of Doxorubicin Peptide Conjugate 1 in Aqueous Solution at 23°C[a]**

pH	Solubility (mg/mL)
2.35	3.98
4.60	95.8
5.72	>156[b]

[a]50 mM citrate buffers were used.
[b]Not completely saturated.

Solution Stability

A pH-rate profile of the doxorubicin peptide conjugate **1** was determined at 40°C at an analytical concentration of 0.2 mg/mL. The solutions were adjusted to the desired pH using 50 mM buffers. The choice of buffers and the observed first-order rate constants (k_{obsd}) are given in Table 2. The observed rate constants are plotted vs. pH in Figure 2, which shows a pH-rate profile for an acid- and base-sensitive compound. The pH of maximum stability of the conjugate is between pH 5 and 6. The pH-rate profile for doxorubicin under the same conditions is also shown in Figure 2. The data indicate that the peptide conjugate of doxorubicin is approximately one order of magnitude less stable than

TABLE 2 **pH-Rate Profile of Doxorubicin Peptide Conjugate 1 at 40°C**

pH	Buffer[a]	$k_{(observed)}$
1.75	HCl/KCl	2.6×10^{-1} h^{-1}
2.99	Citrate	1.5×10^{-2} h^{-1}
4.00	Citrate	1.9×10^{-3} h^{-1}
5.00	Citrate	4.9×10^{-4} h^{-1}
5.70	Citrate	4.3×10^{-4} h^{-1}
5.98	Citrate	5.4×10^{-4} h^{-1}
7.01	Phosphate	4.8×10^{-3} h^{-1}
7.98	Phosphate	7.2×10^{-3} h^{-1}

[a]The total concentrations of the buffers is 50 mM in each case; chloride ion from KCl is used to bring the ionic strength to this level in a 10 mM HCl buffer.

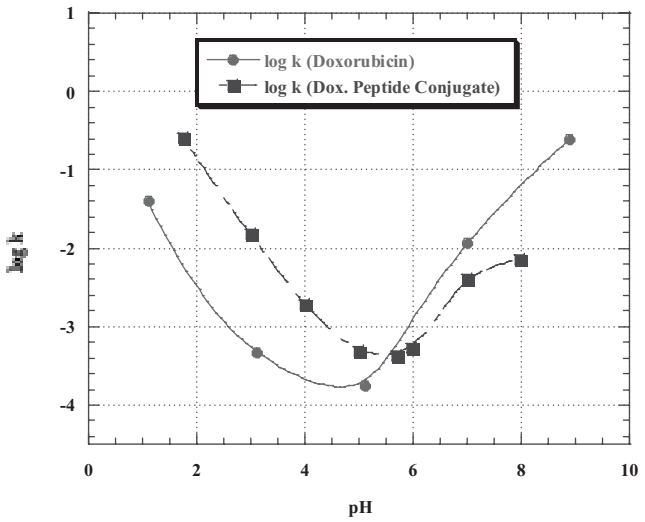
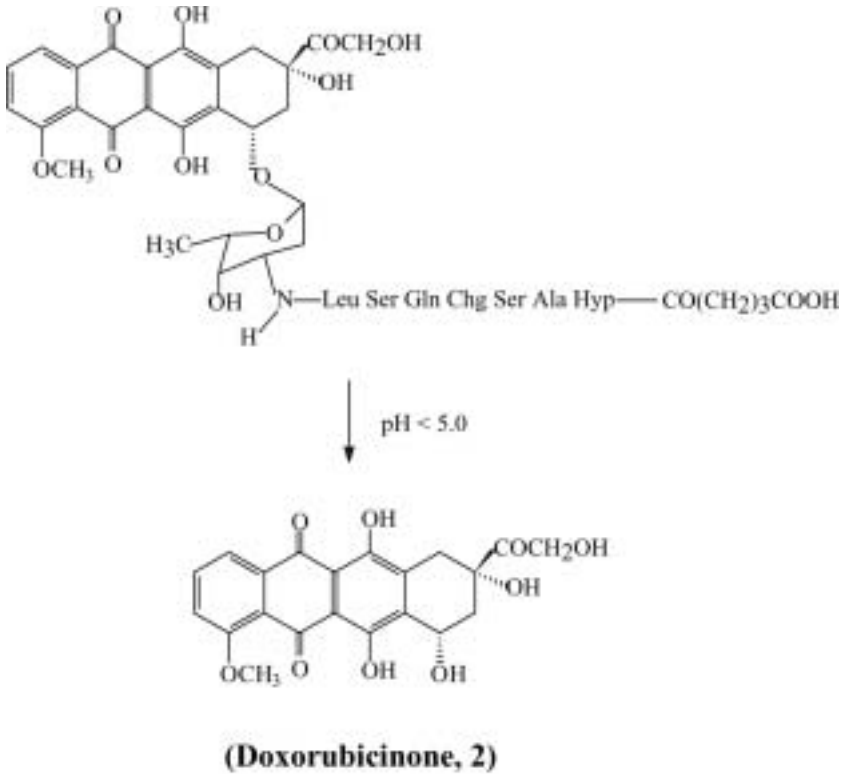


FIGURE 2 **pH-rate profiles for doxorubicin and doxorubicin peptide conjugate 1 at 40°C.**

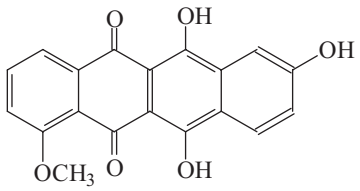
doxorubicin in the acidic range of pH 1–4. The pH-rate profile provides some insight into the degradation pathways for **1**. At pH 5.0 and lower, the major degradation route is cleavage of the sugar moiety as illustrated in Scheme 1. The slope of log k_{obsd} vs. pH is essentially –1.0 in this pH region, supporting specific acid (proton) catalyzed degradation (Beijnen et al., 1985; Wassermann et al., 1983; Beijnen et al., 1987; Beijnen et al., 1986). This effect has been reported in the literature for doxorubicin at pH or 2.1 less (Eigen, 1964). The doxorubicinone (**2**) degradate was isolated and characterization by HPLC-MS gives a molecular ion [M + H] of 415, which is consistent with assignment of the proton catalysis mechanism.

The increased reactivity of **1** relative to doxorubicin at the pH of maximum stability is postulated to result from acylation of the amino-sugar of doxorubicin in **1**, which eliminates the basicity of the $-NH_2$ functional group. In acidic solutions of doxorubicin, the amine is protonated as $-NH_3^+$, which is believed to cause charge repulsion to the proton that is required to be transferred in the transition state of acetal cleavage (Scheme 1), thus slowing down the rate of the reaction. In contrast, acylation of the amino group in the peptide conjugate removes the amine basicity, which will no longer provide destabilization by charge repulsion. Thus, the barrier to the transition state for hydrolysis is lowered, leading to faster degradation of the conjugate compared with doxorubicin. From the data in Figure 3, the second-order rate constant of



(Doxorubicinone, 2)

SCHEME 1 **Chemical structure and degradation pathway for doxorubicin peptide conjugate 1 under acidic conditions.**



(7,8-dehydro-9,10-desacetyldoxorubicinone, 3)

FIGURE 3 **Degradation product of doxorubicin peptide conjugate 1: base catalysis.**

specific acid catalysis, k_H, was calculated to be 1.5×10^{-2} $M^{-1}h^{-1}$ and 1.95 $M^{-1}h^{-1}$ for **1** and doxorubicin, respectively. The roughly 13-fold increase in k_H in **1** compared with doxorubicin is the major contributor to the observed rate at pH 5–6. Similar increases have been observed in the k_{obsd} at the pH of maximum stability of other similar doxorubicin peptide conjugates (data not shown). One notable model compound is Leu-Dox, which is a metabolite of **1** (Dikaola, 2002). The addition of the single amino acid by acylation of the aminosugar decreased the stability of the compound to acid by roughly 10-fold, in good agreement with the quantitative effects on stability of longer-chain peptide conjugates.

To gain further insight into the acid/base catalyzed reactivity of **1** in the intermediate pH range, a study was conducted to assess buffer catalysis at pH 5.3 and 40°C. Buffer catalysis of doxorubicin peptide conjugate was investigated by using several different concentrations of buffer while maintaining constant ionic strength with sodium chloride (0.3 M). The k_{obsd} for the degradation of **1** (Table 3) shows no significant effect of the buffer concentration on the rate constants for degradation. The lack of appreciable buffer catalysis further supports that specific acid catalysis, rather than general acid catalysis, is the dominant mechanism for degradation of the compound in weakly acidic solution.

TABLE 3 **Observed Rate Constants for the Degradation of Doxorubicin Peptide Conjugate 1 at Selected Buffer Concentrations at 3 mg/mL and 40°C[a]**

Buffer concentration (mM)	$k_{(obsd)}$	pH
10 mM	2.3×10^{-4} h^{-1}	5.3
25 mM	2.3×10^{-4} h^{-1}	5.3
50 mM	2.6×10^{-4} h^{-1}	5.2

[a]Including 0.3 M NaCl for constant ionic strength.

Quantitation of the temperature dependence of chemical stability of **1** at the pH of maximum stability (pH 5.7) was done at 25, 40, 60, and 80°C. Figure 4 shows a plot of ln(% Area of Doxorubicin conjugate **1**/% Area reference) vs. time at the four temperatures. The observed first-order rate constant for the loss of **1** was obtained from the experiment, and t_{99} and t_{95} values (time in which 1 and 5% degradation occurs) were calculated (Table 4). The Arrhenius plot of k_{obsd} at the various temperatures is shown in Fig. 5. The apparent activation energy (E_a) is calculated to be 8.4×10^4 J mol^{-1}.

Degradation of **1** at high pH is more complex with various degradation products being observed by HPLC. Structural assignment for one of the late eluting degradates on HPLC, 7,8-dehydro-9,10-desacetyldoxorubicinone (**3**) was suspected, and this compound was previously proposed in the literature for doxorubicin (Beijnen et al., 1986; Abdeen, 1985). Characterization by HPLC-MS gives a molecular ion [M + H] of 337 consistent with the proposed structure. The pH-rate profile is more complex to interpret in the pH range above pH 5.7. There are both oxidative and hydrolytic components, based on the literature for doxorubicin (Wassermann, 1983). Finally, the fate of the relatively nonchromophoric peptide segment remains unclear. Although the peptide contains primary amide (glutamine), there have been no observations of unusual products of acyl rearrangement, for example.

On the basis of the available data, the chemical stability profile of **1** in solution does not support storage at room temperature, a conclusion that is consistent with solubility and physical stability findings (vide

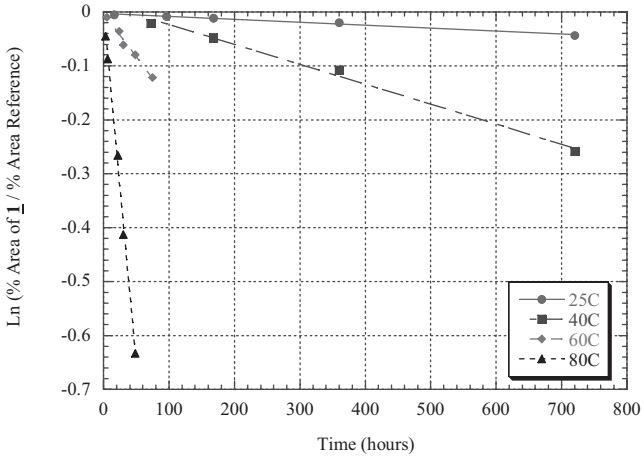


FIGURE 4 **Plot of ln(% Area of Doxorubicin conjugate 1/% Area reference) vs. time (in hours) at 25°C, 40°C, 60°C, and 80°C.**

TABLE 4 **The Rate Constants for Loss of Doxorubicin Peptide Conjugate 1 and Calculated Times to 1% and 5% Conversion as a Function of Temperature[a]**

Temperature	Rate constants (h^{-1})	t_{99} (1% degradation)	t_{95} (5% degradation)
25°C	5.45×10^{-5}	7.7 days	39 days
40°C	3.71×10^{-4}	1.1 days	5.8 days
60°C	1.60×10^{-3}	6.2 h	31.8 h
80°C	1.31×10^{-2}	0.77 h	3.91 h

[a]Solutions of **1** at 50 mg/mL and pH 5.7 (25 mM citrate).

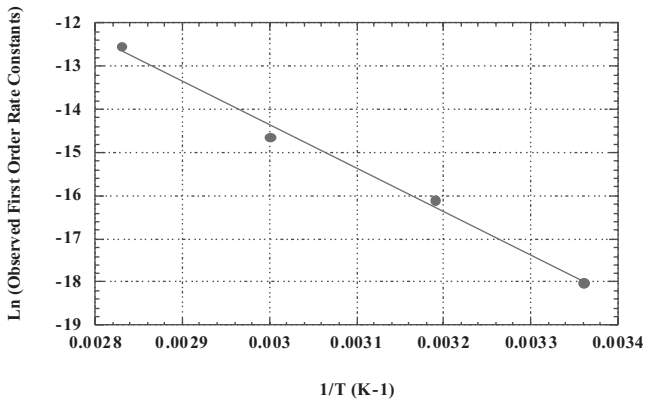


FIGURE 5 **Arrhenius plot for the observed first order rate constants of doxorubicin peptide conjugate 1.**

TABLE 5 **Effect of Sugars on Solution Degradation of 1[a]**

Excipient (%)	First-order rate constant at 40°C (h^{-1})	First-order rate constant at 60°C (h^{-1})
None (buffer only)	5.1×10^{-4}	5.8×10^{-3}
10% lactose	3.7×10^{-4}	5.3×10^{-3}
5% mannitol	4.0×10^{-4}	5.1×10^{-3}
10% sucrose	2.2×10^{-4}	5.4×10^{-3}
5% glucose	2.9×10^{-4}	4.1×10^{-3}

[a]40 mg/mL of **1** in 25 mM citrate pH 5.6 buffer.

supra). Although solutions are too unstable for long-term storage of months to years, prelyophilization concentrates can be made that have sufficient in-process stability. For example, the t_{99} (time for 1% loss by degradation) for a 50 mg/mL solution of **1** in 50 mM citrate buffer at pH 5.7 is 7.7 days at 25°C. The solution of **1** is more stable toward chemical degradation at 5°C, showing a loss of 0.4 Area % in 4 weeks. However, as was found during solubility measurements, the compound forms aggregates in the 7–8 μm range after 4 weeks at 5°C, so that prelyophilization solutions should only be held for brief periods before lyophilization processing. Solutions stored frozen (–20°C) possess acceptable chemical and physical stability, indicating that freeze-concentrate stability will not limit the development of a lyophilized formulation.

Lyophilized Formulations

Chemical stability of **1** is insufficient for a room temperature product for market use, and the physical instability makes it difficult to realize a refrigerated solution product. Because of the chemical and physical instability of solutions of **1**, alternative formulations were considered. A leading option is a lyophilized dosage form for reconstitution. A compatibility study with various pharmaceutically acceptable bulking agents was conducted toward this end. Table 5 shows the chemical stability in solution for a 40 mg/mL solution of **1** in 25 mM citrate buffer at pH of maximum stability (pH 5.6). The data at accelerated conditions (40 and 60°C) show no sign of chemical incompatibility with the sugars. Of particular note was the apparent absence of catalysis by reducing sugars, such as glucose and lactose (Table 5); these would be expected to be problematic in the presence of a free amino group or an enolizable carbonyl function. Because the common sugars did not significantly promote degradation, samples containing 40 mg/mL 1 in 25 mM citrate buffer were lyophilized, and the properties of the lyophilized cakes were compared. Table 6 shows the glass transition temperature (Tg) and the moisture content for the lyophilized products of **1**. Comparison of Tg of various prototype formulations can be made, because the residual moisture levels are fairly constant. On the basis of the thermal data, sugars increase the Tg for the product, and sucrose gives the highest Tg among the excipients tested. Sucrose is a nonreducing sugar and thus provides no opportunity for an aldol reaction with the exocyclic hydroxyacetone function on the anthracycline ring. (This chemistry is

TABLE 6 **Glass Transition (Tg) and Water Content for Lyophilized Formulations of 1[a]**

Formulation	Tg (°C)[b]	Water (%, w/w)
Sucrose (10%)	72.0	0.12
Sucrose (5%)	72.6	ND
Lactose (10%)	53.7	0.02
Lactose (5%)	53.3	0.08
Glucose (5%)	54.0	0.30
Mannitol (5%)	30.2	0.16
No sugar	54.7	0.44

[a]Lyophilized from a solution containing 40 mg/mL of **1** in 25 mM citrate pH 5.6 with sugars at indicated mass percents (w/V in the prelyophilization solutions).

[b]The Tg values are averages of two independent determinations.

a remote possibility and not considered likely in the case of **1**, based on the lack of chemical incompatibility with reducing sugars; see Table 5.) The use of sucrose as a bulking agent for optimal cake properties is, therefore, a rational choice for formulation development.

An optimized formulation was realized on the basis of the use of citrate buffer and sucrose, and the resulting lyophilized product has been tested in clinical studies. The lyophilization process was partially optimized and required less than 2 days of processing to make vials to supply the clinic with acceptable attributes for a product.

CONCLUSION

Doxorubicin peptide conjugate **1** degrades in aqueous solution by specific acid (proton) catalysis at low to intermediate pH. The degradation rate is greater than for doxorubicin, which could be due to acylation of the amino group of the amino-sugar of doxorubicin. Elimination of the positive charge of the protonated amine by acylation appears to favor addition of the specific acid catalyst (a proton) to the ring to promote degradation. The degradation products of the aglycone portion are consistent with the degradation of doxorubicin. Because of the decreased stability of **1** relative to doxorubicin, a lyophilized formulation was necessary for a room temperature product. The formulation design of a viable lyophilized product has been achieved on the basis of physicochemical property considerations and compatibility studies. The resulting prototype formulation has been used in clinical settings.

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