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Stability of Bioreductive Drug Delivery Systems Containing Melphalan is Influenced by Conformational Constraint and Electronic Properties of Substituents

Natthida Weerapreeyakul, R. Gary Hollenbeck and Prashant J. Chikhale*

Department of Pharmaceutical Sciences, 20 North Pine Street, School of Pharmacy, University of Maryland, Baltimore, MD 21201, USA

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Abstract—The stability of bioreductive drug delivery systems (TDDS) was monitored at various pH values and in the presence of glutathione (GSH). Results suggest that steric hindrance due to conformational constraint in TDDS led to an increase in stability of TDDS toward nucleophilic degradation under aqueous conditions. The electronic properties of substituents influenced TDDS stability at different pH values and in the presence of GSH. © 2000 Published by Elsevier Science Ltd.

Bioreductive drug delivery systems containing the methyl ester of melphalan (TDDS) were recently reported to undergo reductive as well as bioreductive activation to catalyze drug delivery.[1–3] Drug delivery via reductive activation of TDDS was shown to be controlled by electronic properties of substituents at the C3′ position.[1] TDDS were designed to be reductively-activated in hypoxic regions of solid tumors, where bioreductive enzymes are known to be overexpressed.[4] Thus under bioreductive conditions, TDDS are reduced to form the unstable hydroquinone intermediate which undergoes spontaneous lactonization (due to conformational constraint in TDDS) to form the corresponding lactone, simultaneously releasing the drug.[1,3] The conformational constraint of TDDS has been attributed to 'trimethyl lock' (a steric interaction between the C6′ methyl and *gem*-dimethyl).[5–8] Further evidence for the conformational constraint of TDDS has also been noted previously during studies involving bioreductive activation of TDDS.[1,3] as well as from crystallographic studies.[9] TDDS were synthesized containing various substituents at position C3′ (e.g., Br-, H-, C_4H_4-, CH_3- or CH_3NH-) on the carrier (quinone) portion in TDDS. The drug portion in TDDS was represented by the methyl ester of melphalan (MME). Recent reports have shown the potential of drug delivery from TDDS as well as from similar molecules, under reductive triggers.[1–3,7–9]

Interestingly, during routine stability tests on TDDS, it was noted that TDDS possessed greater stability compared to the parent compounds, melphalan or MME. Such observations regarding the influence of conformational constraint in drug-carrier systems on the stability of alkylating agents have not been reported. Melphalan was selected as the model antitumor drug.[10] It also possesses good UV detection property, which is useful during stability investigations.[11–14] Clinical inefficacy of melphalan has been reported due to its instability under aqueous hydrolytic conditions and (or) due to glutathione (GSH) conjugation at the bischloroethyl amine portion, thus effectively decreasing the DNA alkylating activity of melphalan.[11–14]

Additionally, GSH conjugated melphalan has been reported to be extruded out of tumor cells via the multidrug resistance associated protein (MRP-1).[14] Thus, stability of TDDS is an important aspect during chemical and biological investigations. Furthermore, stability of TDDS will also be important during biodistribution in order to achieve maximum systemic concentration of TDDS, which in turn, will be useful in providing high alkylating activity at the solid tumor site. Moreover, information from stability studies on TDDS can be useful during proper storage of TDDS samples as well as in the formulation of stable TDDS dosage forms. In this study, we therefore undertook stability studies on TDDS under various pH conditions as well as in the presence of the cellular nucleophile, GSH.

To examine the effect of pH on the stability of TDDS, while controlling the potential influence of ionic strength

*Corresponding author. Tel.: +1-410-706-0739; fax: +1-410-706-0346; e-mail: pchikhal@rx.umaryland.edu; http://www.pharmacy.umaryland.edu/~pchikhal

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and general acid/base catalysis, a buffer system was designed with constant chemical composition and ionic strength. Buffer systems of equal buffer capacity at pH values of 3, 5, 7, and 9 were prepared. A target ionic strength of 0.15 was selected and the design was accomplished by solving a proton balance equation,[15] for a system that included citrate, phosphate and borate to estimate buffer capacity at each of the desired pH values. Once the acid–base chemistry was resolved, the amount of sodium chloride necessary to achieve the target ionic strength was computed. The resulting chemical composition of the buffer system employed was 0.01 M citric acid, 0.02 M phosphoric acid and 0.02 M boric acid, adjusted with sodium hydroxide to the target pH (3, 5, 7, and 9). The physiological, pH 7.4, buffer was prepared as a 0.05 M phosphate buffer solution. TDDS were synthesized using MME as the drug portion to facilitate easy coupling between the bioreductive (quinone carrier) part and MME. The synthesis, purification and characterization of TDDS have recently been published.[1] Stability studies were monitored using an isocratic reverse-phase HPLC system with UV detection (260 nm). HPLC analysis was performed using a Bondclone 10 (C-18) column (300×3.9 mm, 5 μm particle size) with the mobile phase, 65% acetonitrile (HPLC grade)/35% water/0.0135% w/v sodiumdodecylsulfate (as the ion-pairing agent), adjusted to pH 3.05. The flow rate was 1 mL/min with an attenuation of 2 (aufs = 0.01). The HPLC condition was based upon previous reported methods.[11–14] Retention times were 7–13 min for various TDDS, 3.7 min for melphalan, 4.3 min for MME and 4–5 min for corresponding lactones. The minimum detection limit for TDDS was 2 pmol (except for Br-TDDS which was 20 pmol), 30 pmol for the lactones and 30 pmol for MME. All stability assays were reproducible and followed first order kinetics. Time course of degradation and k_{obs} were analyzed by exponential, nonlinear regression of the plot between percentage remaining of the compound versus time. For each TDDS, the time required to reach 50% remaining concentration ($t_{50\%}$) was calculated from the best fit curve ($R^2 > 0.95$). All experiments were run in triplicate.

TDDS were found to possess greater lipophilicity compared to melphalan or MME, according to relative retention as determined by RP-HPLC. The lipophilicity of TDDS was influenced by specific substituents on TDDS in the following order as indicated by their retention on a C-18 column; Br-TDDS (12.5 min) > C_4H_4-TDDS (11.2 min) > CH_3-TDDS (10.9 min) > H-TDDS (8.9 min) > CH_3NH-TDDS (7.6 min) > MME (4.3 min) > melphalan (3.7 min).

Based on the control experiments using HPLC as well as UV spectroscopy (200 to 400 nm) determination at physiological pH 7.4, the parent drugs (melphalan and MME) undergo predominant hydrolysis (data is shown in Table 1). The HPLC chromatograms showed two degradation peaks (mono- and dihydroxylated melphalan), which were consistent with those reported in the literature.[12,16] It is well known that water attacks the aziridinium ring intermediate of melphalan to form the mono- and dihydroxy degradation products.[12,16–17] In earlier studies,[12,16–17] aqueous hydrolysis of melphalan under pH 7.4, 37 °C was reported to occur with the rate of degradation ranging from 0.012 to 0.015 min^{-1}. Our k_{obs} value of 0.014 min^{-1} for melphalan (% remaining = $95.693e^{-0.0141\times time}$, $R^2 = 0.993$) is therefore consistent with the literature.[12,16–17]

During stability studies involving TDDS, under physiological pH 7.4, there was no formation of the lactone or MME, indicating that reduction of TDDS does not occur. TDDS showed approximately 2-fold greater $t_{50\%}$ compared to melphalan or MME at pH 7.4 (Table 1). Interestingly, CH_3-TDDS showed about 10-fold greater stability compared to melphalan or MME. Conformational constraint attributed to 'trimethyl lock' in TDDS seemed to play a role in improving TDDS stability when compared to the parent melphalan or MME. There is no conformational restriction in the structure of melphalan and MME. Further evidence that conformational constraint due to steric interaction between the C6′ methyl group and the *gem*-dimethyl (in the spacer, i.e., side chain) as well as the C3′ functional groups influence TDDS stability, was obtained during pH-stability rate profile studies. Under relatively acidic pH to neutral conditions, all TDDS showed $t_{50\%}$ ranging from 44 to 527 min (data not shown) similar to pH 7.4 stability information reported in Table 1, above. However, in TDDS, in addition to hydrolysis there appears to be nucleophilic attack at the C3′ substituent because the rate of degradation was influenced by the electronic properties of the substituents at the C3′ carbon on quinone ring of TDDS (Table 1). There appears to be a corresponding relation in the electron donating C3′ substituent on TDDS stability even at pH 7.4 (compare the $t_{50\%}$ at pH 7.4 for Br-TDDS and CH_3-TDDS in Table 1). The CH_3NH-TDDS is known to be unstable due to auto-oxidation.[1,7] Therefore at pH 7.4, the degradation of TDDS is most likely a combination of nucleophilic attack at the C3′ position as well as hydrolysis at the bischloroethyl amine portion (similar to that observed in melphalan and MME). It should be noted that half-lives for degradation of TDDS were statistically different ($p < 0.05$) when compared to the parent molecules and that the degradation of TDDS is directly influenced by substituents at the C3′ position in TDDS (Table 1). The markedly greater stability of CH_3-TDDS compared to melphalan at physiological pH is highly interesting. Thus, stability of CH_3-TDDS could prolong

Table 1. Degradation of melphalan, MME and TDDS under physiological pH 7.4, in the presence of glutathione (GSH) and under pH 9, 37 °C as determined using RP-HPLC. Data are mean ± SEM for three determinations

	$t_{50\%}$ (min)		
Compounds	pH 7.4	pH 7.4 + GSH	pH 9.0
Melphalan	46 ± 2	52 ± 0.3	Instantaneous
MME	37±0.1	39±0.3	Instantaneous
Br-TDDS	68±1	< 1	<< 1
H-TDDS	88±0.6	32±1	< 1
C_4H_4-TDDS	92±3	84±2	≈1
CH_3-TDDS	478±17	202±16	6.32 ± 0.09
CH_3NH-TDDS	87±5	134±9	1.22 ± 0.09

physiological availability and hence, biodistribution of CH_3-TDDS.

Melphalan is known to undergo GSH attack at the bischloroethyl amine portion to replace chlorine.[12] Therefore, the degradation of melphalan and MME as well as TDDS was determined in the presence of GSH. HPLC retention profiles of TDDS in the presence of GSH were found to be different from the retention profile of TDDS degradation under pH 7.4 (data not shown). Results therefore indicate that the degradation products (and mechanism) of TDDS in the presence of GSH is different from hydrolytic degradation at pH 7.4. As shown in Table 1 above, when TDDS were subjected to GSH-catalyzed degradation, C_4H_4-, CH_3-, and CH_3NH-TDDS exhibited greater stability, compared to the GSH-catalyzed degradation of the parent melphalan or MME. The CH_3-TDDS was also found to be the most stable in the presence of GSH, similar to observations at pH 7.4 (Table 1). Apparently, GSH attack is also predominantly catalyzed by the electron-withdrawing characteristic of the C3′ substituent on TDDS. As a result, Br-TDDS and H-TDDS underwent a faster rate of glutathionyl conjugated degradation (shorter $t_{50\%}$) than the parent drug (melphalan and MME) due to the ease of the attack of GSH at the C3′ position, which bears Br- or H-substituents. If GSH attacked only at the bischloroethyl region in TDDS, then all TDDS would have been observed to degrade at similar rates. Instead, TDDS showed GSH-catalyzed degradation according to the electronic properties of the C3′ substituent. Therefore, we conclude that GSH attack was predominantly at position C3′ and was influenced by electronic properties of the substituents at position C3′ (compare Br- and H-TDDS with C_4H_4- and CH_3-TDDS in Table 1). GSH attack at the bischloroethyl amine portion of TDDS is also likely similar to the report of Bolton et al.[12] regarding glutathione conjugation with melphalan.

We used UV spectroscopic studies from 200 to 400 nm in addition to HPLC based-degradation studies to determine hydrolysis degradation of melphalan and MME under pH 9. Melphalan and MME (similar to TDDS) showed maximum absorbance at 260 nm. Furthermore, maximum absorbance of the hydroxylated degradation products of melphalan (mono- and dihydroxylated melphalan) was found to be at the same region (260 nm). The UV wavelength region (260 nm) was reported previously for detection of the hydroxylated melphalan products.[12,16,17] Thus, it is possible that one can monitor degradation at the bischloroethyl amine portion at 260 nm. UV spectra of melphalan and MME degradation under pH 9 did not show any new formation of other degradation product. The HPLC profiles of melphalan degradation studies showed two degradation peaks of hydroxylated melphalan (e.g., mono- and dihydroxylated melphalan). MME degradation studies at pH 9 also showed similar peaks attributed to hydroxylated products of melphalan indicating that MME degradation resulted in melphalan, which subsequently underwent hydroxylation at the bischloroethyl amine. The observation of melphalan hydroxylation at pH 9 was consistence with melphalan hydroxylation at pH 7.4 in our studies reported here as well as with previous reports.[12,16,17] Results shown in Table 1 above indicate that melphalan and MME undergo very rapid degradation at pH 9 when compared to degradation at pH 7.4. MME shows slightly more degradation, which could be attributed to two different degradation pathways; hydrolysis degradation at the bischloroethyl amine region (pathway no. 1 in Fig. 1) and hydrolysis of the methyl ester (pathway no. 4 in Fig. 1). On the other hand, melphalan is degraded only by hydrolysis at the bischloroethyl region.

TDDS demonstrated spontaneous degradation at the highly basic pH (pH 9) leading to formation of two major degradation products as identified by HPLC (Table 1 and Fig. 2). The degradation product peaks of TDDS at pH 9 were distinguishably different than those

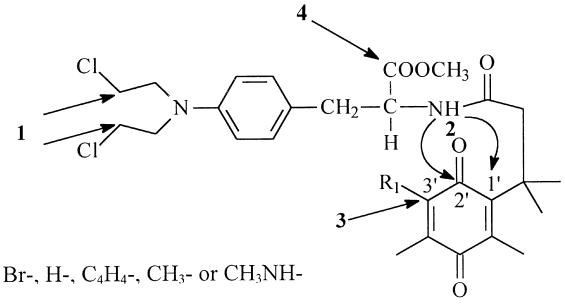

Figure 1. TDDS structure and four possible degradation sites on TDDS. (**1**) Hydrolysis at the bischloroethyl amine portion; (**2**) intramolecular cyclization because of the attack of the reactive amide 'nitrogen' at position C2′ or C1′; (**3**) substituent effect at C3′ for nucleophilic attack on the C3′ 'carbon'; and (**4**) degradation by hydrolysis of the methyl ester.

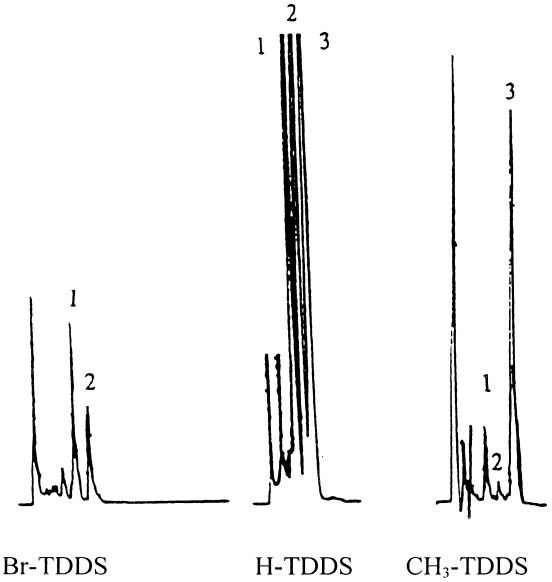

Figure 2. Representative HPLC chromatograms of Br-, H- and CH_3-TDDS during stability investigation at pH 9, 37 °C. The mobile phase was 65% acetonitrile−35% H_2O with 0.0135% w/v SDS, pH 3.05 ($\lambda = 260$ nm). All samples were taken at 1 min. The two major degradation products of TDDS (1 and 2) were formed at different rates as indicated by the chromatographic peaks clearly demonstrating substituent effect on rate of TDDS degradation at pH 9.

at pH 7.4. Two degradation peaks of TDDS at pH 9 were prominent. These results indicate that the degradation mechanisms were different between pH 7.4 and pH 9. Therefore at pH 9, predominant degradation of TDDS occurs via pathway no. 2 (Fig. 1). Our data thus indicate that TDDS formation has resulted in insignificant degradation at the bischloroethyl region of MME, when conjugated to form TDDS. However, underivatized melphalan and MME are prone for hydrolysis at the bischloroethyl portion, consistent with the literature. Br-TDDS and the unsubstituted H-TDDS showed the fastest rate of degradation at pH 9. On the other hand, TDDS containing electron-donating substituents (e.g., C_4H_4-TDDS and CH_3-TDDS) exhibited a relatively slower rate of degradation at the basic pH. Therefore, the result suggest that electron-withdrawing properties of the C3′ substituent tend to decrease the stability of TDDS at pH 9, which can be attributed to nucleophilic attack at position C3′ on TDDS.

Previously, it was reported that structures resembling TDDS, particularly quinone propionic amide containing *p*-anisidine underwent degradation to form a 6-membered ring, hydroxy dienone and a 5-membered ring, spirolactam (due to attack of the nucleophilic nitrogen amide at either the C2′ or C1′ position on the quinone ring).[18,19] At pH 9, TDDS could undergo a similar degradation route. The two degradation products for each TDDS showed a consistent retention time difference of approximately 2 min. However, depending on the substituents on TDDS, the peak area 'ratio' for the two major degradation products (between the 'fast' and the 'slow' eluting degradation peaks) differed significantly and ranged from 2.8 to 0.8 min (Fig. 2). These observations (at alkaline pH) indicate that electronic properties of substituents at position C3′ on TDDS affect rate of formation of the two major degradation products as well as the rate of nucleophilic attack at C3′ (Fig. 1 and Table 1). Based on the observed HPLC chromatograms (Fig. 2), identification and characterization of the two major degradation products were attempted by collecting fractions of each degradation product using HPLC followed by NMR and MS (FAB and EI). We made numerous attempts to purify the degradation products by HPLC. However, most likely due to instability of degradation products, we were unable to isolate the degradants of TDDS at pH 9.

Therefore, we also used UV spectroscopy from 200 to 400 nm in an attempt to identify the degradation products of TDDS under pH 9 (Fig. 3). The benzoquinone propionic acid, melphalan and MME were also compared (as control) to TDDS. Based on HPLC studies, H-TDDS was selected for the UV spectroscopic studies due to its fast rate of degradation yielding high amounts of the two major degradation products. H-TDDS (10.4 μM) in acetonitrile was used as a control. Changes in UV absorption as a function of H-TDDS stability at pH 9 was recorded. An H-TDDS sample (20.47 μM) in pH 9 buffer solution, 37 °C was prepared. The H-TDDS (0.5 mL) sample was taken at 1 and 10 min and each sample was diluted with 0.5 mL of acetonitrile for the UV spectrophotometric determinations. During UV spectrophotometric studies, it was observed that H-TDDS ($\lambda_{max} = 259$ nm) rapidly underwent degradation (denoted by (a), (b) and (c) in Fig. 3) resulting in a peak at $\lambda = 232$ nm. A similar bathochromic shift was earlier reported for the quinone propionic acid amide containing *p*-anisidine, suggesting formation of the hydroxy dienone.[19] It should be noted that although at 1 min, both degradation products (1 and 2) of H-TDDS were observed by HPLC (Fig. 2). The 232 nm peak (Fig. 3) could be attributed to the major degradant (peak no. 1) since at later time points, only the degradant (peak no. 1) was shown to be the major product. Such intramolecularly catalyzed degradation was suggested to be a result of the steric constraint created by the 'trimethyl lock' configuration.[18,19] Therefore the degradation of TDDS at pH 9 is influenced by conformational constraint created by steric interaction between *gem*-dimethyl and C6′ methyl, which results in close placement (proximity) of the amide 'nitrogen' poised for intramolecular attack at C2′ and C1′ positions in TDDS. In addition, TDDS degradation at pH 9 is also significantly influenced by the electronic effects of the substituents at the C3′ position (as shown in Table 1 and discussed above).

As a control, the stability of benzoquinone propionic acid at pH 9 was monitored by RP-HPLC and by using UV spectroscopy (maximum absorbance at 255 nm). The benzoquinone propionic acid was found to be stable at pH 9. UV scans of benzoquinone propionic acid at pH 9 did not show the 232 nm peak, which was observed during TDDS degradation at pH 9. Thus, we determined that the quinone portion is stable by itself at pH 9. Therefore, these observations provide further evidence for the most likely C2′ and C1′ intramolecular attack in TDDS to generate 232 peak(s) at pH 9.

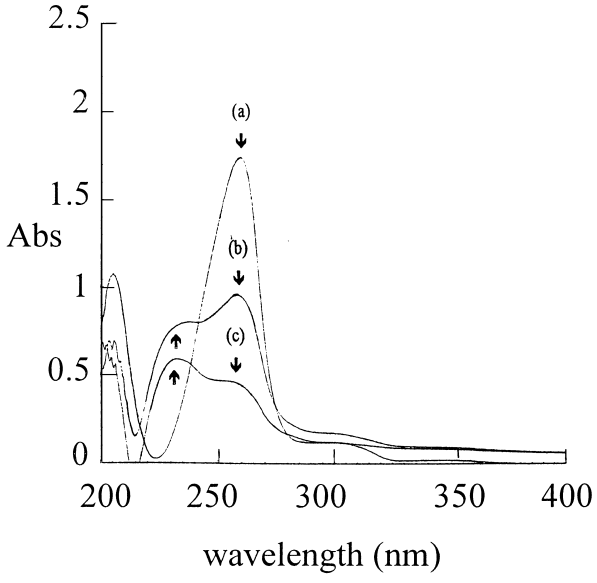

Figure 3. Degradation of H-TDDS at pH 9, 37 °C as monitored using UV spectroscopy (200 to 400 nm) demonstrating bathochromic shift of H-TDDS from $\lambda_{max} = 259$ nm to $\lambda = 232$ nm. (a) H-TDDS in acetonitrile as control (10.4 μM); (b) H-TDDS stability sample at 1 min and (c) H-TDDS stability sample at 10 min.

However, when the quinone is conjugated to the drug part via an amide linkage, two predominant degradation reactions occur: intramolecular attack of the amide 'nitrogen' at C2′ or C1′ (see Figs. 2 and 3) and the nucleophilic attack at C3′ (see Table 1).

In conclusion, at pH 7.4, TDDS undergo hydrolytic degradation at the C3′ position on TDDS and possible hydrolysis at the bischloroethyl amine portion (see Fig. 1). Apparently, the C3′ substituted 'carbon' is susceptible to nucleophilic attack as seen by the substituent effect at the C3′ position in TDDS (compare Br-, H-, C_4H_4-, and CH_3-TDDS in Table 1). On the other hand, at pH 9 there is pronounced degradation of TDDS attributed to intramolecular attack of the reactive amide 'nitrogen' of the side chain at the C2′ and C1′ position in TDDS (Fig. 1) and the substituent effect at C3′ for nucleophilic attack on the C3′ 'carbon'. It is important to note that degradation product peaks for TDDS as observed under pH 9 were different than degradation product peaks observed under physiological pH. The overall observed degradation rates of the TDDS under pH 7.4 were less than those observed rates under pH 9 (Table 1). The conformationally constrained quinone carrier was therefore found to decrease nucleophilic-based degradation at the bischloroethyl amine portion of TDDS structure, compared to the parent melphalan or MME, under pH 7.4 as well as at pH 9. The glutathionyl conjugation-based degradation of TDDS also occurred at position C3′ of the quinone ring due to the substituent effect (as observed for Br- and H-TDDS). Among the TDDS, electronic properties of substituents at the C3′ position clearly influenced TDDS stability in the presence of GSH, under physiological pH (7.4) as well as under basic pH (9) conditions. In general, electron-donating substituents at C3′ provided relatively greater stability to TDDS compared to stability of the parent melphalan or MME under the same conditions. Interestingly, CH_3-TDDS was found to be the most stable compound and TDDS of the type, CH_3-, C_4H_4- and CH_3NH- may not be susceptible for GSH-catalyzed, MRP-1 mediated extrusion from tumor cells. Furthermore, stability of the CH_3-TDDS will be useful during systemic circulation and its biodistribution in the treatment of solid tumors. Thus, stability of CH_3-TDDS and its resistance to GSH-based degradation would likely provide greater tumor concentration of CH_3-TDDS during bioreductive activation in solid tumors.

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