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Targeted drug delivery systems 6: Intracellular bioreductive activation, uptake and transport of an anticancer drug delivery system across intestinal Caco-2 cell monolayers☆

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Abstract

We demonstrate transport across, intracellular accumulation and bioreductive activation of a conformationally constrained, anticancer drug delivery system (the CH_3-TDDS) using Caco-2 cell monolayers (CCMs) as an *in vitro* model of the human intestinal mucosa. Reverse-phase High Performance Liquid Chromatography (HPLC) coupled with UV detection was used to detect CH_3-TDDS, the bioreduction product (lactone) and the released drug (melphalan methyl ester; MME). Upon incubation of the CH_3-TDDS with the apical (AP) surface of 21-day-old CCM, we observed rapid decrease in the AP concentration of the CH_3-TDDS (60%/hr) as a result of cellular uptake. Rapid intracellular accumulation of the CH_3-TDDS was followed by bioreductive activation to deplete the cellular levels of CH_3-TDDS. The drug part (MME) and lactone, as well as CH_3-TDDS, were detected in the basolateral (BL) chamber. Intracellular Caco-2 levels of TDDS and lactone were also detectable. Bioreductive activation of the CH_3-TDDS was additionally confirmed by formation of lactone after incubation of the CH_3-TDDS in the presence of freshly prepared Caco-2 cell homogenates. During transport studies of melphalan or MME alone (as control), the intact drug was not detected in the intracellular compartment or in the BL chamber. These observations demonstrate that CH_3-TDDS has potential for improving intestinal delivery of MME. TDDS could be useful in facilitating oral absorption of MME as well as the oral delivery of other agents.

Keywords: Anticancer drug delivery system; Caco-2; Intestinal transport; Intracellular bioreductive activation; Melphalan; Uptake

1. Introduction

Recently, numerous reports have emerged on the use of conformationally constrained prodrug systems (Amsberry and Borchardt 1991; Gharat et al., 1998; Wang et al., 1999; Greenwald et al., 2000; Killian et al., 2000; Killian and Chikhale,

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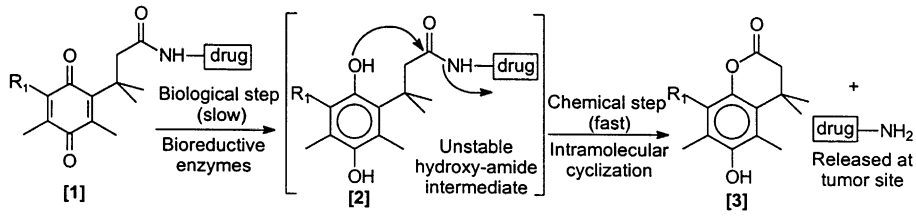

Fig. 1. Principle of drug delivery from the CH_3–TDDS **[1]**. Bioreduction of CH_3–TDDS ($R_1 = CH_3$) to form the unstable hydroquinone intermediate **[2]** is followed by rapid cyclization to generate the lactone **[3],** consequently releasing the antitumor agent. In this study, the antitumor drug portion is the methyl ester of melphalan (MME).

2001; Weerapreeyakul et al., 2000, 2000a). The prodrug delivery systems (TDDS) were shown to be reductively activated as well as bioreductively activated to catalyze drug delivery under conditions similar to those present in solid tumors (Chikhale et al., 1997; Gharat et al., 1997; Gharat et al., 1998; Weerapreeyakul et al., 2000a). Bioreductive activation of the TDDS **[1]** to form an unstable hydroquinone intermediate **[2]** is followed by a rapid intramolecular cyclization reaction, resulting in formation of the lactone **[3]**, simultaneously releasing MME (the drug part) (Fig. 1). One of the candidate TDDS, the CH_3–TDDS (where $R_1 = CH_3$) was recently shown to possess greater stability than the parent molecules (melphalan and MME) (Weerapreeyakul et al., 2000). Potentially, the CH_3–TDDS could be useful in improving intestinal drug delivery. Therefore, in this report, we determined intestinal cell uptake of CH_3–TDDS, intracellular accumulation and bioreductive activation, followed by transport of the CH_3–TDDS across intestinal epithelium by using Caco-2 cell monolayers (CCMs) as an in vitro model of the human intestinal mucosa (Fig. 2).

The human adenocarcinoma cell line Caco-2, which was originally isolated from a human colon adenocarcinoma by Fogh et al. (1977), has been developed as a model of the intestinal epithelium (Grasset et al., 1984; Hidalgo et al., 1989). This cell line has been extensively used to study intestinal transport of a variety of agents (Hilgers et al., 1990; Artusson, 1990). Studies on concurrent transcellular transport and intracellular metabolism of drug molecules using CCMs are also described in literature (Chikhale and Borchardt, 1994; Walter and Kissel, 1994). Since TDDS have recently been observed to be susceptible to bioreductive activation, we determined bioreductive activation of the CH_3–TDDS during transport across Caco-2 cell monolayers. Conversion of TDDS to MME by bioreductive enzymes should occur in the intracellular compartment of Caco-2 cells (as shown in Fig. 2).

2. Materials and methods

2.1. Materials

The Caco-2 cell line originating from a human colorectal carcinoma was obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagles Media (DMEM) was

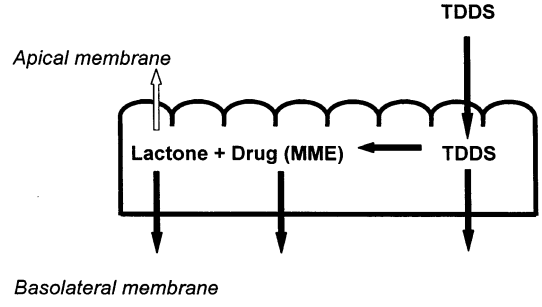

Fig. 2. Schematic representation of concurrent transport across, and intracellular bioreductive activation of CH_3–TDDS in, Caco-2 cell monolayers (CCMs).The apical uptake of TDDS is followed by bioreductive activation to catalyze drug delivery (MME release) with simultaneous formation of lactone in the intracellular compartment of Caco-2 cells. The TDDS, lactone and MME diffuse across the BL membrane. Lactone was also detected in the AP chamber of Transwells®.

obtained from Sigma Chemical Co. (St. Louis, MO). Non essential amino acids (NEAAs), fetal bovine serum (FBS), Ethylenediaminetetraacetic acid (EDTA)/trypsin and penicillin/streptomycin sulfate solution were purchased from Biofluids Inc. (Rockville, MD). Transwells® clusters (3.0 μm pore size; 4.71 cm^2 surface area) were purchased from Corning–Costar (Cambridge, MA). Radiolabeled ^{14}C-mannitol (specific activity of 51 mCi/mmol) was obtained from DuPont NEN (Boston, MA). The CH_3–TDDS **[1]** and MME were synthesized, purified and characterized in our laboratory (Gharat et al., 1998). Stock solutions of CH_3–TDDS (1–2 mM) and MME (6–7 mM) in acetonitrile were used for transport studies. All solvents used to prepare the mobile phase were of HPLC grade.

2.2. Methods

2.2.1. HPLC assay

All samples were analyzed by reversed phase HPLC, using a C18 column and ion-pairing conditions. The HPLC system (Shimadzu) consisted of LC-10AS pump, LC-10A controller, SIL-10A auto-injector and CR501 chromatopac integrator. The mobile phase was a mixture of 65% acetonitrile and 35% water containing 0.0135%(w/v) sodium dodecylsulphate, adjusted to pH 3.05 using 1 N HCl. The flow rate was 1.0 ml/min with an injection volume of 10 μl. All samples were monitored with a SPD-10A UV-vis detector set at 260 nm (0.01 aufs). The limits of detection were 0.03 nmol for MME, 0.03 nmol for the CH_3–lactone, and 0.002 nmol for the CH_3–TDDS **[1]**. The retention time was 7.8 min for the CH_3–TDDS, 2.3 min for the corresponding CH_3–lactone **[3]** and 14 min for MME. The assay was linear and reproducible and was based on the previously described method of Adair et al. (1984).

2.2.2. Caco-2 cell culture

Caco-2 cells were grown in culture using conditions similar to those reported earlier (Hidalgo et al., 1989; Chikhale and Borchardt 1994). Briefly, Caco-2 cells were plated and grown at 37°C in T-75 flasks in an atmosphere of 5% CO_2 and 95% relative humidity, using DMEM supplemented with 10% FBS, 1% NEAAs and 0.5% penicillin/streptomycin. Before reaching confluency, the Caco-2 cells were trypsinized with 0.02% EDTA and 0.05% trypsin. The cells were then plated on polycarbonate Transwells® membranes (3.0 μm pore size) at a seeding density of 90,000 cells/cm^2. These cells were cultured for 21 days during which the media was changed every second day. All transport and metabolism studies were performed in 0.05 M phosphate buffer, pH 7.4, at 37°C and the transwells were lightly stirred (50 oscillations/min).

2.2.3. Caco-2 cell monolayer integrity

Caco-2 cell monolayer integrity was determined by measuring transepithelial electrical resistance (TEER) and ^{14}C-mannitol permeability. TEER values for the control cell monolayers (incubated under 0.05 M phosphate buffer) and cell monolayers treated with 50 μM CH_3–TDDS were determined at room temperature using a Multicell-ERS resistance system obtained from Millipore Corporation. TEER values were corrected for the blank (polycarbonate membranes without the Caco-2 cell monolayers). Mannitol transport was examined in the apical to basolateral direction for a duration of 3 h. CCM integrity studies were conducted under condition similar to those used during transport studies, as appropriate controls. ^{14}C-Mannitol containing 0.05 M phosphate buffer, pH 7.4 (1 ml) was applied to apical side. The basolateral chamber contained phosphate buffer (1.5 ml). At 30, 60, 90, 120, 150 and 180 min, each transwell was moved to a new chamber containing fresh phosphate buffer (1.5 ml). Samples (0.5 ml) were added to 5 ml scintillation cocktail and counted using a scintillation counter (Beckmann Instruments).

2.2.4. Transport and intracellular uptake studies in Caco-2 cell monolayers

In order to examine the potential of CH_3–TDDS for intracellular delivery, the intracellular uptake and transport of CH_3–TDDS across confluent Caco-2 cell monolayers (CCMs) were determined. The CCMs used in these studies were between passages 35 and 53, and were 21 days

old. After reaching confluency, the apical (AP) and the basolateral (BL) surfaces of the cell monolayers were washed three times with fresh 0.05 M phosphate buffer, pH 7.4. The CH_3–TDDS (50 μM) in phosphate buffer (1 ml) was placed on the apical surface of the cell monolayer. Phosphate buffer (1.5 ml) was placed in the basolateral chamber. Buffer-volume was maintained at a minimum level to avoid excessive dilution. Transwells® containing CH_3–TDDS in phosphate buffer solution in the AP side was incubated at 37°C for various time periods. Each well in Transwells® was used for only one time point in the determination of AP, BL, and cellular level of the TDDS and the products of bioreductive activation. Samples (500 μl) were collected from the apical and the basolateral chambers of each transwell at the proper time point (t = 0, 20, 60, 110 and 170 min) and diluted with ice-cold acetonitrile (500 μl) before HPLC analysis. The control experiments were carried out to determine nonspecific binding of the CH_3–TDDS to the Transwells® filters.

For determination of concurrent intracellular bioreductive activation of the CH_3–TDDS during transport across CCMs, the individual cell monolayers were washed with fresh phosphate buffer (three times) at various time points (0, 20, 60, 110 and 170 min). The cells were scraped off from the Transwells® with a rubber spatula and collected in 1 ml of 0.1 N HCl in microcentrifuge tubes. The cell suspension was homogenized by ultrasonication under ice for 15 s at a low intensity. The cell homogenate (200 μl) was mixed with ice-cold acetonitrile (200 μl) and centrifuged (15,000 g) for 5 min. The clear supernatant (10 μl) was analyzed by HPLC for the presence of CH_3–TDDS and the products of bioreductive activation of CH_3–TDDS (i.e., MME and lactone **[3]**).

2.2.5. Bioreductive activation of CH_3–TDDS in Caco-2 cell homogenates

To determine whether CH_3–TDDS undergoes bioreduction during transport across Caco-2 cell monolayers, we confirmed bioreductive activation of CH_3–TDDS using freshly prepared Caco-2 cell homogenates. The apical (AP) and the basolateral (BL) surfaces of the cell monolayers were washed three times with fresh 0.05 M phosphate buffer, pH 7.4. The cells were scraped off the Transwells® with a rubber spatula and collected in microcentrifuge tubes using 1 ml phosphate buffer. The cell suspension was homogenized under an ice-bath for 15 s by ultrasonication (at a low intensity), and then warmed to 37°C. CH_3–TDDS (50 μM) was mixed into the cell homogenate. Samples (50 μl) were withdrawn at 0, 5, 10, 20, 40 and 60 min, mixed with ice-cold acetonitrile (50 μl) and centrifuged (15,000 g) for 5 min. The supernatant was analyzed by HPLC for the presence of CH_3–TDDS, MME and lactone **[3]**.

3. Results and discussion

3.1. Caco-2 cell monolayer integrity

The TEER values for confluent Caco-2 cell monolayers grown onto polycarbonate membranes in Transwells® measured using 0.05M phosphate buffer were greater than 940 Ω cm^2 at room temperature. The TEER values for confluent Caco-2 cell monolayers treated with 50 μM CH_3–TDDS in 0.05 M phosphate buffer showed less than 4% deviation from the control values. In addition, the amount of ^{14}C-mannitol transported across the confluent Caco-2 cell monolayers was determined to be $< 1\%$ in 2 h [$P_m = 0.791(\pm 0.004) \times 10^{-6}$ cm/s] indicating that the cell monolayers were intact during the course of studies. These results show that the Caco-2 cell monolayers retained their integrity throughout the experiments.

3.2. AP Caco-2 cell membrane permeability of CH_3–TDDS

The flux (5.30×10^{-6} μmol/s) of CH_3–TDDS across the AP membrane of the CCM was calculated based on the disappearance of CH_3–TDDS from the AP chamber. The AP membrane permeability (2.25×10^{-5} cm/s) was then calculated using the following equation;

$$P_m(\text{cm/s}) = \frac{\text{Flux}}{A \times C_O}$$

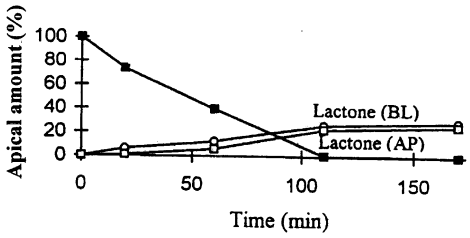

Fig. 3. Time profile for concurrent intestinal transport across and bioreductive activation of CH_3–TDDS **[1]** in CCMs.TDDS (50 μM) in 0.05 M phosphate buffer (1 ml) (pH 7.4, 37°C) was placed on the AP surface of confluent CCMs in Transwells®. Phosphate buffer (1.5 ml) was placed in the BL chamber. During transport studies, samples (500 μl) were taken from AP and the BL chambers at 0, 20, 60, 110 and 170 min and mixed with 500 μl ice-cold acetonitrile, for HPLC analysis of TDDS, lactone and MME. The rate profile shows rapid cellular uptake of TDDS from the AP chamber and the percentage lactone released on the AP and the BL sides, as determined by HPLC. Lactone is formed only after bioreductive activation of the intracellular TDDS, thus leading to the release of MME. Error bars, representing standard error of the mean value, are smaller than the symbols in the graph.

where A is the surface area of the monolayer (4.71 cm^2) and C_o is the initial donor concentration (0.05 μmol/ml). In control studies, it was determined that the CH_3–TDDS does not bind to Transwells®. Furthermore, the filter membranes (3.0 μm pore size) did not provide significant resistance to diffusion of the TDDS from the AP to BL compartment in control Transwells® (i.e., without the CCMs).

3.3. Transport, uptake and bioreductive activation of CH_3–TDDS in Caco-2 cell monolayers

Upon incubation of the CH_3–TDDS on the AP surface of confluent CCMs, we observed a rapid uptake (60%/hr) of CH_3–TDDS across the AP membrane in CCMs (see Fig. 3). The AP uptake of CH_3–TDDS was found to have a $t_{1/2}$ of 56 ± 4 min (Table 1) and the AP membrane permeability in CCM was determined to be 2.25×10^{-5} cm/s. Thus, CH_3–TDDS was rapidly accumulated within the Caco-2 cells. The intracellular CH_3–TDDS was bioreductively activated to form the lactone **[3]**, consequently releasing the drug portion (MME). Thus, the accumulated intracellular CH_3–TDDS was depleted mainly due to bioreductive activation. As much as 8% CH_3–TDDS and 8% lactone **[3]** were detected intracellularly within 1 h, indicating fast uptake across the AP cell membrane and a relatively slow rate of bioreduction of CH_3–TDDS by CCM (Fig. 4). The lactone formed as a result of bioreductive activation in the Caco-2 cells was eliminated almost equally, across the BL membrane and the AP membrane (~25% eliminated on each side of the CCM) (Fig. 3). Therefore, a total of about 40% lactone could not be accounted for. This 40% loss of lactone could be attributed to its intracellular metabolism. It should be noted that the lactone has a phenolic-OH functional group, and in our

Table 1
Bioreductive activation of CH_3–TDDS in Caco-2 cell homogenates, cellular uptake and apical Caco-2 cell membrane permeability to CH_3–TDDS.

Caco-2 cell Homogenate $t_{1/2}$ (min)[a]	AP Uptake Rate $t_{1/2}$ (min)[b]	AP Permeability cm/s[c]
138 ± 6	56 ± 4	2.25×10^{-5}

[a] Rate of bioreductive-activation of CH_3–TDDS (50μM) per mg protein, in the presence of Caco-2 cell homogenate.
[b] Uptake rate of CH_3–TDDS across AP membrane of confluent Caco-2 cell monolayers in culture.
[c] Apical Caco-2 cell membrane permeability for CH_3–TDDS.

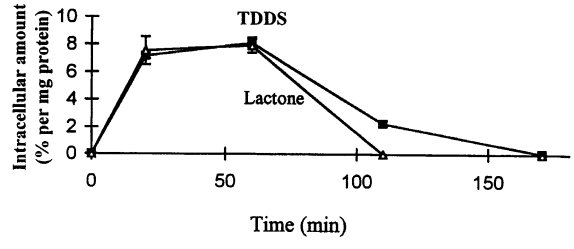

Fig. 4. Time profile for intracellular accumulation of CH_3–TDDS and formation of lactone due to intracellular bioreductive activation of the TDDS during transport of the TDDS across confluent CCMs.Percentage TDDS and lactone, as determined by HPLC, in the intracellular compartment of confluent CCMs in Transwells® during concurrent intestinal transport and bioreductive activation of TDDS (50 μM). MME was not detected in the Caco-2 cells. However, the chemical degradation product of MME was observed (not shown).

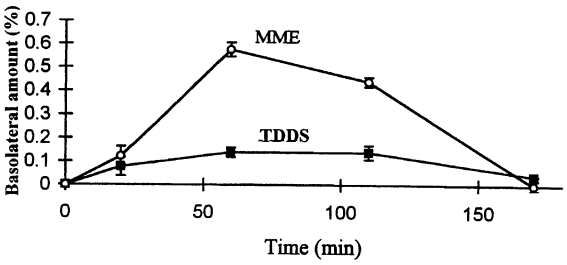

Fig. 5. Time profile for basolateral levels of CH_3–TDDS and released MME during transport of the TDDS across confluent CCMs.Percentage TDDS and MME as determined by HPLC in the BL chamber of Transwells® during concurrent intestinal transport and bioreductive activation of the TDDS (50 μM) in CCMs.

separate control experiments involving the in vitro incubation of the lactone with glucuronic acid and glucuronosyl-transferase, the lactone was observed to degrade. This observation is consistent with reports on glucuronidation as well as the capacity of Caco-2 cells to catalyze sulfation (Bjorge et al., 1991; Chikhale and Borchardt, 1994; Abid et al., 1995; Prueksaritanont et al., 1996). A small proportion of CH_3–TDDS and the released drug portion (MME) was transported to the basolateral side (Fig. 5). It is quite likely that the metabolic shunting in Caco-2 cells due to intracellular bioreductive activation, results into a "sink condition", serving as the driving force for the AP concentration of CH_3–TDDS. We noted that the MME released from CH_3–TDDS during intracellular bioreductive activation underwent chemical degradation. The chemical degradation of MME was recently reported under similar conditions, which has been attributed mainly to hydrolysis of the bischloroethyl amine portion of MME, similar to the degradation of melphalan (Stout and Riley 1985; Weerapreeyakul et al., 2000). Based on the combined percentages of the lactone (AP + BL + intracellular) detected it can be concluded that nearly 100% of CH_3–TDDS is taken up by the Caco-2 cells within 2 h (Figs. 2–5), with a significant fraction of the TDDS accumulating within the cells (8–10%). A major fraction of TDDS undergoes intracellular bioreductive activation (60%). Approximately 30% of the CH_3–TDDS could not be accounted for, most likely due to initial fast bioreductive activation of CH_3–TDDS subsequent to uptake into the CCMs, followed by loss of lactone due to intracellular conjugation in addition to loss of MME due to degradation. There was no detectable loss of the TDDS in the Transwells® filter due to nonspecific adsorption. Therefore, the uptake process, metabolism and permeability of the TDDS are intrinsic.

During AP–BL studies, it was evident that TDDS was rapidly taken up in the CCM. The high level of bioreductive activation was attributed completely to the intracellular Caco-2 cell compartment as observed from the high level of lactone formation. Bioreductive activation acts as a sink-mechanism to drive high levels of the TDDS into Caco-2 cells. CH_3–TDDS rapidly disappeared from the apical side during the transport studies. In control studies, no bioreductive activation was observed in the AP and BL compartments. TDDS and lactone are stable in both the AP and BL compartments during the transport studies, as determined in separate control stability studies in the phosphate buffer. All of this evidence strongly suggests the unidirectional movement of TDDS, predominantly from the AP to the BL direction. In our other sets of studies involving the blood–brain barrier (membrane), we have also observed that TDDS shows predominantly high levels of diffusion (Killian et al., 2000; Killian and Chikhale, 2001). Since the TDDS decreased rapidly from the AP compartment, we did not undertake BL–AP studies. There was no indication of an efflux mechanism for the TDDS. We anticipate that if efflux was involved, significant amount of TDDS would have been detected in the AP chamber in Transwells®.

During transport and uptake studies of MME and melphalan alone (as control studies) across confluent CCMs, we observed rapid chemical degradation of MME and melphalan on the AP side of the CCM. The chemically degraded MME and melphalan was detected in Caco-2 cells as well as on the BL side in Transwells®. Intact MME and melphalan could not be detected in the cells during these (control) transport studies. Furthermore, the CH_3–TDDS (in separate control

studies) was highly stable and was found not to undergo bioreductive metabolism in the buffer matrix, which was preincubated with the CCMs (Weerapreeyakul et al., 2000). Therefore, these results indicate that the CH_3–TDDS improved intracellular as well as transcellular delivery of MME across confluent CCMs, compared to the parent molecules, MME or melphalan.

Melphalan (*p*-[*bis*(chloro-2-ethyl)amino]-L-phenylalanine), an alkylating agent, was introduced in the late 1950s and has since been established as an agent with a wide spectrum of antitumor activity (Sarosy et al., 1988; Sameuls and Bitran, 1995). It is extensively used in the treatment of multiple myeloma, ovarian cancer, breast cancer, neuroblastoma, regionally advanced malignant melanoma and localized soft-tissue carcinoma (Sameuls and Bitran, 1995). The alkylating activity of melphalan is due to formation of interstrand or intrastrand DNA cross-links or DNA-protein cross-links via the two chloroethyl groups on melphalan (Furner and Brown, 1980). A major problem with oral administration of melphalan is its variable bioavailabilty (Choi et al., 1989). The problem of melphalan bioavailability is further complicated by its rapid hydrolysis (degradation) at physiological pH (Evans et al., 1982) and high plasma protein binding (Gera et al., 1989). Melphalan is actively transported into the cells by the high-affinity L-amino acid transport system which also transports the amino acids, glutamine and leucine (Vistica, 1979). Hence, when melphalan is administered orally with food, its absorption is reduced. It has been shown that melphalan transport across the intestinal mucosa is inhibited by the structurally related amino acids present in the ingested food (Adair and McEnlay, 1987; Reece et al., 1986).

Due to problems with oral administration, variation of bioavailability, instability and high protein binding of melphalan, as mentioned above, a drug delivery system to improve the intestinal absorption of melphalan, will be useful. Thus, derivatization of melphalan to change its transport route from an active process (carrier-mediated) to a passive process (diffusion) is considered to be a useful strategy in improving the oral absorption properties of melphalan. Dipeptide derivatives of melphalan have been shown to enhance accumulation of melphalan in Caco-2 cells (Kupczyk-Subotkowska et al., 1997). These dipeptide derivatives were passively transported through the apical membrane of Caco-2 cells due to their high lipophilicity and their overall neutral charge of the ester, and subsequent intracellular formation of the more polar amino acids (Kupczyk-Subotkowska et al., 1997). Dipeptide-like strategies have been reported for L-Dopa (Bodor et al., 1977) as well as for melphalan and acivicin (Killian et al., 2000). Transient blocking of the amino acid functional group in melphalan and acivicin by coupling to the drug delivery system (the CH_3–TDDS) was recently reported to change the large neutral amino acid (LNAA) mediated transport of the parent anticancer agents across the blood–brain barrier (BBB) into the passive mechanism of brain uptake (Killian et al., 2000; Killian and Chikhale, 2001). Acivicin derivatives blocked on either the carboxy- or the amino- terminus of the L-α-amino acid led to removal of recognition for the LNAA transporter at the BBB (Chikhale et al., 1995). The CH_3–TDDS is most likely passively transported into cells and across membranes, since the quinone propionic acid carrier moiety imparts lipophilicity to the TDDS (Killian and Chikhale 2001; Weerapreeyakul et al., 2000). Furthermore, rapid uptake of the CH_3–TDDS into Caco-2 cells, and subsequent intracellular bioreductive activation (Fig. 3 and Fig. 4), is consistent with the lipophilic nature of CH_3–TDDS. It should be noted that the amide bond between the quinone moiety and the drug part (MME) of TDDS is stable. Therefore, the stability of CH_3–TDDS in the AP chamber could also contribute to high availability of the TDDS in Caco-2 cells.

3.4. Bioreductive activation of CH_3–TDDS in Caco-2 cell homogenate

The presence of bioreductive enzymes in colon cancer cell lines, as well as in colorectal tumor samples, is well-documented in the literature (Mikami et al., 1996; Smitskamp-Wilms et al., 1995; Smitskamp-Wilms et al., 1996; Mikami et al., 1998). Thus, bioreductive activation of CH_3–

TDDS was demonstrated by incubation of CH_3–TDDS with Caco-2 cell homogenate. Upon incubation of CH_3–TDDS with the homogenates, the drug delivery system was bioreductively activated at a pseudo first-order rate, as given by $t_{1/2}$ (138 ± 6 min) (Table 1). Bioreductive activation was confirmed by formation of lactone **[3]** (Fig. 6). Incidentally, MME released from CH_3–TDDS undergoes rapid chemical degradation, and therefore could not be detected (Fig. 6). We reported earlier that MME undergoes degradation by hydrolysis at the bischloroethyl amine side chain (Weerapreeyakul et al., 2000). In these studies, the major degradation product of MME was detected at the retention time of 4.5 min, which was similar to our observations during control stability studies on MME in phosphate buffer, pH 7.4 at 37°C (data not shown). The degradation of MME is also consistent with observations during its transport across CCMs, as discussed above in Section 3.3.

In the presence of Caco-2 cell homogenates, the rate of bioreduction of CH_3–TDDS ($t_{1/2} = 138 \pm 6$ min) was slower than its AP uptake rate across CCMs ($t_{1/2} = 56 \pm 4$ min) (Table 1). This data supports our observation of rapid uptake of CH_3–TDDS across the AP surface of CCMs, and the high level of accumulation of TDDS inside the Caco-2 cells, followed by the rate-limiting, intracellular bioreductive-activation in Caco-2 cells. The thermodynamic driving force for intracellular penetration of CH_3–TDDS appears to be very high, followed by a relatively slow rate of "metabolic shunting" due to bioreductive activation of CH_3–TDDS in the Caco-2 cells. As a result, the CH_3–TDDS tends to form a depot (reservoir) within the Caco-2 cells for slow (sustained) intracellular bioreductive activation, leading to intracellular drug delivery. Therefore, we confirmed that CH_3–TDDS is susceptible to bioreductive activation in the presence of reductive enzymes in the intracellular compartment of Caco-2 cells. Thus, TDDS could serve as a useful delivery system for improving intestinal drug delivery and absorption.

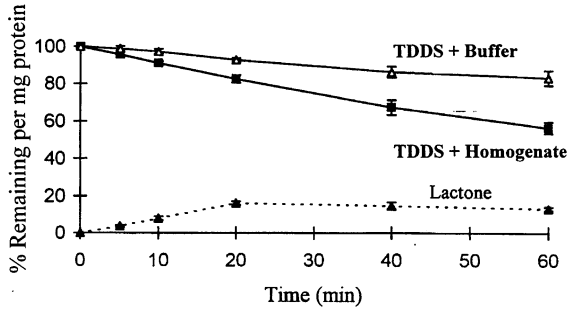

Fig. 6. Bioreductive metabolism of TDDS in Caco-2 cell homogenates.Caco-2 cell homogenate samples (50 μl) spiked with TDDS (50 μM) were taken at 0, 5, 10, 20, 40 and 60 min, mixed with 50 μl ice-cold acetonitrile and centrifuged (15000 g) for 5 min under ice-cold conditions. The supernatant was analyzed by HPLC for TDDS, MME and lactone. Error bars represent standard error of the mean value for three determinations.

4. Conclusion

We used the human adenocarcinoma cell line, Caco-2, to determine concurrent transport and intracellular bioreductive metabolism of CH_3–TDDS. The uptake rate of CH_3–TDDS from the AP side was observed to be faster than the "metabolic shunting" rate of bioreductive activation of CH_3–TDDS in Caco-2 cells. Thus, CH_3–TDDS accumulates within Caco-2 cells to create a reservoir for sustained, intracellular release of the drug via bioreductive activation of the TDDS. It should be noted that Caco-2 is a human colon adenocarcinoma cell line (Fogh et al., 1977). Colon cancer cell lines have been shown to overexpress bioreductive enzymes, particularly DT–diaphorase (Mikami et al., 1996; Smitskamp-Wilms et al., 1996). The presence of bioreductive enzymes (i.e., microsomal cytochrome P450 reductase) within Caco-2 cells has also been recently reported (Rossi et al., 1996). Our results showed that CH_3–TDDS undergoes efficient bioreduction within the Caco-2 cells during concurrent transport across CCMs, resulting in drug delivery within the cytosol of Caco-2 cells as well as in the basolateral chamber. CH_3–TDDS improved the delivery of MME across CCMs when compared to the parent, underivatized molecules, melphalan or MME. Therefore, CH_3–TDDS has good potential to deliver MME

as well as other agents across the intestinal epithelium during oral absorption.

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