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Reformulation of etoposide with solubility-enhancing rubusoside

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ABSTRACT

Etoposide (ETO), a widely used anti-cancer drug, is constrained by its low aqueous solubility and by side effects from both the drug and its solubilizing excipients. In this study, a recently discovered natural solubilizer rubusoside (RUB) was used to achieve the solubilization of ETO. Dynamic light scattering and freeze-fracture transmission electron microscopy studies showed that ETO and RUB formed ETO–RUB nanoparticles (~6 nm in diameter). The powder of ETO–RUB nanoparticles was completely reconstitutable in water and remained stable in this solution at 25 and 37 °C for at least 24 h. Under other physiologic conditions, ETO solution was clear and free of precipitation at 25 °C, but underwent various structural transformations. In PBS and simulated intestinal fluid, RUB-solubilized ETO underwent epimerization and equilibrated to *cis*-ETO. In simulated gastric fluid, RUB-solubilized ETO degraded to 4′-demethylepipodophyllotoxin-beta-D-glucoside and 4′-demethylepipodophyllotoxin. Higher temperatures favored epimerization or degradation. Furthermore, a side-by-side comparison with DMSO-solubilized ETO confirmed that the RUB-solubilized ETO showed no significant differences in cytotoxicity in colon, breast and prostate cancer cell lines. RUB effectively solubilized and stabilized etoposide, which sets the stage for further toxicology, bioavailability, and efficacy investigations.

1. Introduction

Etoposide (ETO, Fig. 1), a semi-synthetic derivative of the natural lignan podophyllotoxin, is an old yet still widely used chemotherapeutic drug. It inhibits the enzyme topoisomerase II that is involved in the unwinding of the DNA molecule during replication. In clinical applications, this standard chemotherapy treats diverse types of cancers such as Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme (Henwood and Brogden, 1990; IARC, 2000).

Although the US Food and Drug Administration (FDA) approved three commercial formulations of etoposide for clinical intravenous (*i.v.*) and oral administration, many problems hamper the desired therapeutic efficacy. Etoposide is poorly soluble in water, which explains the use of many excipients such as polysorbate 80, polyethylene glycol, and alcohol in the injectable solution. Furthermore, these formulations must be diluted to the concentration of 0.2–0.4 mg/mL to avoid precipitation (Schacter et al., 1994). Large doses of etoposide may, therefore, occasionally require administration of significant fluid volumes. This fluid load can cause heart failure in some patients and presents an obstacle to rapid administration of etoposide and to long-term home infusion administration regimens (Hande, 1998). Adverse effects such as hypotension, anaphylaxis, and bronchospasm may also occur with rapid administration of etoposide (O'Dwyer and Weiss, 1984; Ogle and Kennedy, 1988), perhaps due to the solubilizing ingredients in the formulation (Varma et al., 1985). Etoposide phosphate, a prodrug of etoposide, solves the solubility problem and eliminates the need for using problematic solubilizing ingredients but requires biotransformation to release the active moiety. Although etoposide phosphate is bioavailable at an average level of 50%, the variation in bioavailability is high (25–75%), which is undesirable for consistent clinical efficacy. The oral administration of etoposide increases variability in bioavailability both within and between patients as compared to the intravenous injection. Joel et al colleagues tried to improve the oral bioavailability and narrow the variability in bioavailability of etoposide by concomitant administration of a plethora of excipients including ethanol, bile salts, cimetidine, metaclopromide and propantheline without much success (Joel et al., 1995a). This high variability in oral bioavailability may involve the activity of P-glycoprotein (Pgp), of which etoposide is a substrate. Preclinical studies suggest that inhibition of Pgp may improve the bioavailability of etoposide (Leu and Huang, 1995).

Many approaches have been examined to improve the formulation of etoposide. Existing pharmaceutical formulation techniques have been employed for etoposide including nanoparticle-based

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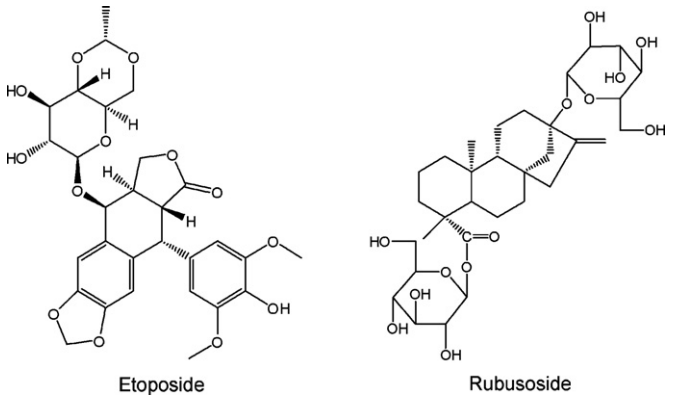

Fig. 1. The structures of etoposide and rubusoside.

delivery systems (Yadav and Sawant, 2010), liposomal delivery systems (Sengupta et al., 2000), micelles complexes and phospholipid-based microemulsion (Reddy et al., 2006; Jain et al., 2010). Although each has made advancements, fundamental and practically significant improvement remains an elusive goal.

In a search to identify bioactive compounds from plant sources, it was discovered that some steviol glycosides such as stevioside, rebaudioside A, and rubusoside possess solubilizing properties (Liu, 2009). Rubusoside (RUB, Fig. 1), in particular, showed solubility enhancement for curcumin (Zhang et al., 2011) and paclitaxel (Jeansonne et al., 2011). Rubusoside is a commonly known natural sweetening agent and its major use is seen in food and beverage products. In this study, we developed a novel etoposide formulation, in which rubusoside acted as a solubilizer. The RUB-solubilized etoposide formulation was characterized for its solubility enhancement, loading efficiency, and particle size along with its stability in physiological solutions. Furthermore, the RUB-solubilized etoposide formulation was evaluated for its anti-cancer potential in human colon, breast, and prostate cancer cell lines.

2. Materials and methods

2.1. Materials

Acetonitrile and water were of HPLC grade (Mallinckrodt Baker Inc., Phillipsburg, NJ). Formic acid (98%) was of HPLC grade (Sigma–Aldrich Co., St. Louis, MO). Absolute ethanol was of ACS/USP grade (Pharmco-AAPER Manufacturer, Brookfield, CT). Phosphate buffered salts were purchased from MP Biomedicals, LLC (Solon, OH). Simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8) were purchased from RICCA Chemical Co. (Arlington, TX). Etoposide with purity of 98% was purchased from LKT Laboratories, Inc. (St. Paul, MN). Rubusoside was isolated from *Rubus suavissimus* S. Lee (Rosaceae) in our own laboratory (Liu, 2009) and structurally elucidated by NMR and MS analyses. The purity of rubusoside was determined to be above 98% by HPLC–UV. Uranyl acetate was purchased from TED PELLA Inc. (Redding, CA). DMSO was of analytical grade (Fisher Scientific Inc., Fair Lawn, NJ). The solution of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate were purchased from Promega Co. (Madison, WI).

2.2. HPLC and HPLC–MS protocols

An HPLC system (Waters, Milford, MA) was used for the analyses. The system included a pump, an on-line degasser, a column heater compartment, an auto-sampler, and a photodiode array (PDA) detector. Empower[1] workstation software was used for the control of the equipment, acquisition and processing of data. All of the analyses were performed on a reversed-phase Symmetry C18 HPLC column (150 mm long × 4.6 mm i.d.; 5 μm) at 30 °C. For ETO and RUB detection, elution was performed with an isocratic gradient of acetonitrile and water containing 0.02% formic acid (32:68). The flow rate of the mobile phase was 1.0 mL/min and the injection volume of sample was 2 μL. The PDA detector was in a wavelength range of 200–600 nm and the selected wavelength was 285 nm for ETO detection and 215 nm for RUB detection. For transformation detection of ETO, elution was performed with an isocratic gradient of acetonitrile and water containing 0.02% formic acid (25:75). The flow rate of the mobile phase was 1.0 mL/min and the injection volume of sample was 2 μL. The PDA detector was in a wavelength range of 200–600 nm.

HPLC–MS system included a pump, an on-line degasser, a column heater compartment, an auto-sampler, a photodiode array (PDA) detector and an EMD1000 mass spectrometer with an ESI interface and a TOF mass analyzer (Waters, Milford, MA). Empower[2] workstation software was used for the control of the equipment, acquisition and processing of data. All of the analyses were performed on a reversed-phase Phenomenex Luna C18 column (150 mm long × 2.0 mm i.d.; 5 μm) at 30 °C with a mobile phase of acetonitrile and water (26:74) at 0.30 mL/min. The sample injection volume was 2 μL. Elution from HPLC was introduced into a mass spectrometer with an ESI interface and the data were collected with the following settings: a negative scan, 3.5 kV capillary, 25.00 V cone, 1.00 V extractor, 120 °C source temperature, 400 °C desolvation temperature, 50 L/Hr cone gas flow (N_2) and 400 L/Hr desolvation gas flow (N_2), and full scan mode (*m/z* 200–900).

2.3. Preparation of ETO–RUB powder

Appropriate amounts of RUB and ETO were weighed and mixed at the ratio of 10:1 w/w. Then, 10 mL of ethanol was added to the mixture, vortexed slightly, and heated in a water bath for 5 min to form a clear ethanol solution. The ethanol solution was passed through 0.45 μM nylon filters (Whatman, Maidstone, UK) to eliminate large particles present in the solution. The ethanol suspension was allowed to stand in the room temperature for 60 min. The ethanol in the suspension was then evaporated under reduced pressure, heat at 50 °C, and agitation in a RAPIDVAP system (Labconco, Kansas City, MO). The resulting RUB–ETO powder was reconstituted with HPLC grade water and centrifuged at 14,000 rpm for 10 min. The supernatant was analyzed for ETO and RUB.

2.4. Reconstitution of ETO–RUB powder in water

Appropriate amounts of ETO–RUB powder were weighed (Table 1) and water was added. After vortex, all solutions were centrifuged for 10 min at 14,000 rpm and the supernatants were analyzed by HPLC and a pH meter (Fisher Scientific Inc., Pittsburgh, PA). The concentrations of ETO were determined using a series of ETO standard solutions in methanol between 60 and 5000 μg/mL. The concentrations of RUB were determined using a serial of RUB standard solutions in water between 1 and 100 mg/mL.

2.5. Characterization of the solubilized structures between ETO and RUB

2.5.1. Determination of surface morphology

Surface morphology of ETO–RUB nanoparticles was determined by freeze-fracture transmission electron microscopy (FF-TEM). Freeze-fracture samples were prepared by first depositing a drop of 10% (w/v) RUB-solubilized ETO water solution onto a copper planchette (BAL-TEC, Los Angeles, CA). The sample was frozen by rapid immersion into a liquid Freon (SHUR/Freeze™ Cryogen Spray, Triangle Biomedical Sciences, Durham, NC) bath, then

Table 1
Solubility of etoposide in water solutions reconstituted from the ETO–RUB powder.

Sample[a]	ETO–RUB added (mg)	H_2O added (mL)	RUB detected in solution (mg/mL)	ETO detected in solution (μg/mL)	pH
ETO–RUB 0.1%	5.01 ± 0.02	5.0	0.66 ± 0.02	80.85 ± 3.37	4.72 ± 0.03
ETO–RUB 0.25%	5.00 ± 0.03	2.0	2.09 ± 0.02	210.54 ± 3.20	4.80 ± 0.01
ETO–RUB 0.5%	5.00 ± 0.02	1.0	4.41 ± 0.02	411.36 ± 5.00	4.92 ± 0.05
ETO–RUB 0.75%	7.51 ± 0.02	1.0	6.74 ± 0.04	618.16 ± 8.35	5.04 ± 0.04
ETO–RUB 1%	10.04 ± 0.03	1.0	9.02 ± 0.05	826.77 ± 10.35	5.18 ± 0.03
ETO–RUB 2.5%	25.02 ± 0.02	1.0	22.78 ± 0.02	2058.85 ± 5.18	5.54 ± 0.04
ETO–RUB 5%	50.04 ± 0.03	1.0	44.61 ± 0.15	4100.67 ± 34.81	5.86 ± 0.07
ETO–RUB 7.5%	75.04 ± 0.04	1.0	65.38 ± 0.08	6247.58 ± 33.07	5.92 ± 0.08
ETO–RUB 10%	100.07 ± 0.03	1.0	85.23 ± 0.28	8457.74 ± 94.61	6.02 ± 0.02

[a] Each sample was measured in three replicate experiments and expressed as mean ± SD ($n = 3$).

plunged in liquid nitrogen. The vitrified sample was transferred to the sample stage, which was submerged in liquid nitrogen. After transferring the sample stage into the freeze-etching chamber of the Balzers BAF-400 apparatus, the samples were fractured at a temperature of −170 °C. Once the fractured sample was allowed to etch for ~1 min, Pt–C was deposited at a 45° angle with respect to the sample surface followed by deposition of C at a 90° angle for reinforcement. The resulting replicas were washed twice in distilled water to remove the actual sample. The replicas were collected on 400 mesh Formvar-coated grids (Electron Microscopy Sciences, Hatfield, PA) and allowed to dry overnight. TEM observations were performed with a JEOL JEM-100CX transmission electron microscope operated at 80 kV.

2.5.2. Particle size measurement

Particle size measurements were performed using a custom-built apparatus equipped with a Coherent Innova 90 argon (400–800 nm) laser set to 568.2 nm. A Pacific Precision Instruments wide-range photometer/preamplifier/discriminator drives an ALV pulse shaper, which feeds an ALV-5000 digital autocorrelator. RUB-solubilized ETO water solution (10%, w/v) was transferred into clean cells via 0.22 μm Millipore Durapore Membrane filters. The temperature was controlled at 25 °C by a circulating water bath. For each sample, five repetitive runs, each of 180 s duration, were collected at 90° scattering angle. The averaged correlation functions were analyzed with one-exponential and third-order cumulant algorithms to determine the apparent hydrodynamic diameter, D_h.

2.6. Stability profiles of RUB-solubilized ETO in physiologic solutions

2.6.1. Stability over time

The ETO–RUB powder was reconstituted in three replicates in water (control), PBS solution, simulated gastric fluid, and simulated intestinal fluid, respectively. The reconstituted solutions were stored at 37 °C or 25 °C. The concentrations of ETO and RUB in the reconstituted solutions were measured at 0, 2.5, 5, 7.5, 10, 12.5 and 24 h by HPLC following sample preparation. Results were expressed as mean ± SD (standard deviation).

2.6.2. Stability in response to dilution

The ETO–RUB powder was reconstituted to stock solutions in three replicates in water (control), PBS solution, simulated gastric fluid, and simulated intestinal fluid, respectively. Each reconstituted stock solution contained 2.5% (w/v) RUB and was then diluted by 2.5, 5, or 10 fold with the same dissolving solvent. All solutions were stored at 37 °C or 25 °C. The concentrations of ETO and RUB in the diluted solutions were measured at 0, 2.5, 5, 7.5, 10, 12.5 and 24 h by HPLC following sample preparation. Results were expressed as mean ± SD.

2.7. Anticancer activity of RUB-solubilized ETO

2.7.1. Solubilization and sample preparation

Two ETO stock solutions were prepared by either solubilizing the free ETO in absolute DMSO (ETO–DMSO) or reconstituting the ETO–RUB powder in water to a concentration of 10.2 mM. Both stock solutions were diluted with culture medium by 100 fold, which became the assay stock solutions. All assay stock solutions were serially diluted by either culture medium with (for ETO–DMSO) or without (for ETO–RUB) 1% (v/v) DMSO.

2.7.2. Cell culture

The human prostate carcinoma (PC3), breast carcinoma (MDA-MB-231), and human colon adenocarcinoma (HT-29) cell lines were obtained from the American Type Culture Collection (ATCC) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, HEPES, penicillin-streptomycin, sodium pyruvate, L-glutamine, and non-essential amino acids. All cell culture materials were purchased from Invitrogen Corporation (Carlsbad, CA).

In vitro cytotoxicity assays were conducted using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. HT-29, PC3, and MDA-MB-231 cells were added to 96-well plates at 1×10^4 cells/well, respectively, and allowed to adhere overnight. The cells were then treated with various ETO solutions prepared above ranging from 0.8 μM to 102.0 μM in triplicate wells and incubated at 37 °C for 72 h. On day three, a 20 μL aliquot of MTS solution premixed with phenazine methosulfate was added directly to each well and the plate was incubated at 37 °C for another 1 to 2 h. Absorbance was measured at a wavelength of 490 nm using a Bio-Rad Microplate Reader (Hercules, CA). Percent viability was calculated as cell viability relative to vehicle-treated control (100%). The IC_{50} values were the average of at least two independent experiments.

All data were analyzed using Student's *t*-test (SAS, Cary, NC) and the means were compared for each cell line. Significance of all tests was set at $P \leq 0.05$ and data were expressed as mean ± SE (standard error).

3. Results

3.1. Solubility enhancement of ETO by RUB

HPLC was used to detect ETO and RUB in this study. The selected wavelength for quantifying ETO and RUB was chosen at 285 nm and 215 nm, respectively. The retention times of ETO and RUB were 5.2 and 8.3 min, respectively, under the elution scheme described in Section 2. As Table 1 shows, ETO at 81 μg/mL went into water solution in the presence of 0.7 mg/mL RUB. When the RUB concentration increased to 85 mg/mL (the highest in this study), the solubility of

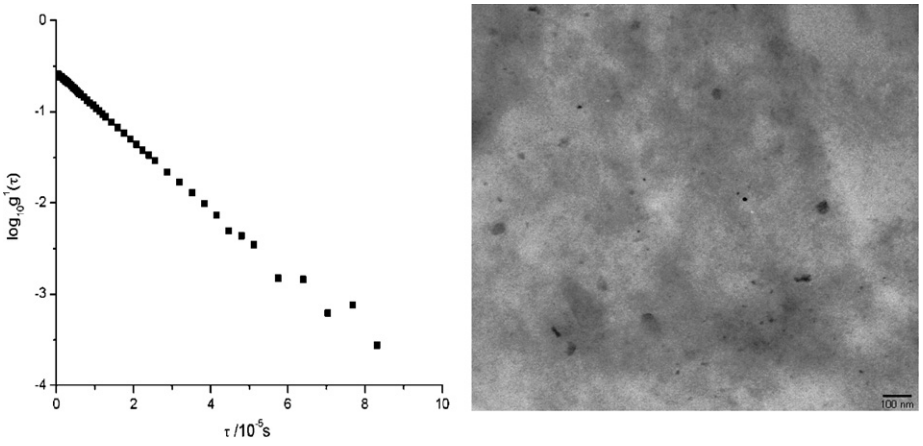

Fig. 2. (A) Semilogarithmic plot of normalized first-order autocorrelation function for 10% (w/v) RUB-solubilized etoposide water solution. (B) FF-TEM image (direct magnification 80,000×) of ETO–RUB nanoparticles in the 10% RUB-solubilized ETO water.

ETO in water increased to 8458 μg/mL, the highest concentration achieved in this study. A linear relationship was found between the concentrations of ETO and RUB, which was *Y* (ETO concentration in μg/mL) = 97.69 × (RUB concentration in mg/mL) − 58.10, R^2=0.9986. Based on the linear model, it was predicted that every 1 mg/mL increase in RUB level within the experimental range of 10–100 mg/mL would bring additional 97.69 μg/mL of ETO into the water solution. RUB concentrations were stable in all RUB-solubilized ETO water solutions. The pH of the RUB-solubilized ETO solutions ranged from 4.7 to 6.0, corresponding to the increasing trend of both RUB and ETO concentrations.

3.2. Characterization of ETO–RUB structures

Based on the linear behavior of the semilogarithmic plot shown in Fig. 2A, the particles are confirmed as monodispersed. The measured apparent hydrodynamic diameter, D_h, for the ETO–RUB was 6.3 ± 0.6 nm. Although conclusive evidence about the size and dispersity of the solution was obtained from the semilogarithmic plot of the autocorrelation function, further distribution analyses (e.g., by the popular Laplace inversion method CONTIN) reaffirmed these findings. Fig. 2B shows FF-TEM images of a 10% RUB solubilized ETO water solution. The many dark spots were particles formed between ETO and RUB in the nanometer range, in agreement with the DLS results.

3.3. Stability and dilutability of nanoparticulate ETO under physiological conditions

3.3.1. Stability over time

The nanoparticulate ETO–RUB powder was completely reconstituted in water, PBS solution, simulated gastric fluid, and simulated intestinal fluid, respectively. These reconstituted solutions contained approximately 23 mg/mL of RUB and 2 mg/mL of ETO. At 25 °C, ETO in the reconstituted ETO–RUB water solution was physically and chemically stable for at least 24 h (Fig. 3A). The relative standard deviation of ETO concentrations in 24 h was less than 0.8%. Under other physiologic conditions, however, ETO underwent various epimerization or degradation. The concentration of ETO decreased over 24 h at 25 °C by 4% in PBS solution (pH 7.4), 13% in simulated gastric fluid (pH 1.0), and 2% in simulated intestinal fluid (pH 6.0), respectively. At 25 °C, there was no observed physical instability. In contrast, at 37 °C, physical instability, i.e., suspension at first and precipitation subsequently was observed visually in each of the three reconstituted solutions (Fig. 3B–D). Besides physical instability in the form of precipitation, chemical instability in the forms of structural transformations or degradation of ETO in PBS solution, simulated intestinal fluid, and simulated gastric fluid may have also taken place, contributing to an overall greater decrease of ETO concentrations at 37 °C than at 25 °C.

To characterize the degradation products, each reconstituted ETO–RUB physiologic solution was kept at 37 °C for 20 h to induce degradation. Each resultant solution was then analyzed by HPLC–MS (Fig. 4). In the water solution where no degradation took place, ETO was the single peak identified at 12.8 min with *m*/*z* at 587 ($[M-H]^-$) matching the standard. In the PBS solution and simulated intestinal fluid, in addition to ETO, another peak was detected at 16.7 min with *m*/*z* at 587 ($[M-H]^-$). Based on the UV and MS characteristics, this peak was identified as the *cis*-lactone of etoposide. In the simulated gastric fluid, other than the ETO, two new peaks appeared at 3.5 and 9.4 min with *m*/*z* at 561 and 399 ($[M-H]^-$), respectively. They had similar UV spectra as that of ETO, but the molecular weight (MW) was 16 and 188 Daltons less, respectively. Considering the structure, synthesis of ETO, and acidic solvent, it was concluded that ETO degraded to two products of 4′-demethylepipodophyllotoxin-beta-D-glucoside (MW 562) and 4′-demethylepipodophyllotoxin (MW 400). HPLC analysis indicated that about 13% of ETO was degraded in the simulated gastric fluid in 24 h, which was quicker than its equilibration to epimerization in PBS solution or simulated intestinal fluid. In contrast to the physical and chemical instability shown in ETO, the solubilizing RUB itself was physically and chemically stable under all physiologic conditions shown above for ETO.

3.3.2. Stability in response to dilution

The ETO–RUB powder was completely reconstitutable in water, PBS solution, simulated gastric fluid, and simulated intestinal fluid to form the respective stock solutions. These reconstituted stock solutions contained approximately 23 mg/mL of RUB and 2 mg/mL of ETO. The reconstituted stock solutions were then diluted by a factor of 2.5, 5, and 10 with respective aqueous medium. It was found that all diluted solutions were stable during the dilution process and free of precipitation. ETO in the diluted water solution was chemically stable at 25 °C and 37 °C, respectively (Fig. 3A) with a less than 2% change over a 24-h period. Under other physiologic conditions, the same epimerization or degradation was observed in the diluted solutions as in the reconstituted stock solutions. Furthermore, higher temperature, such as 37 °C, favored epimerization or degradation of ETO than 25 °C (Fig. 3B–D) in the diluted physiologic solutions.

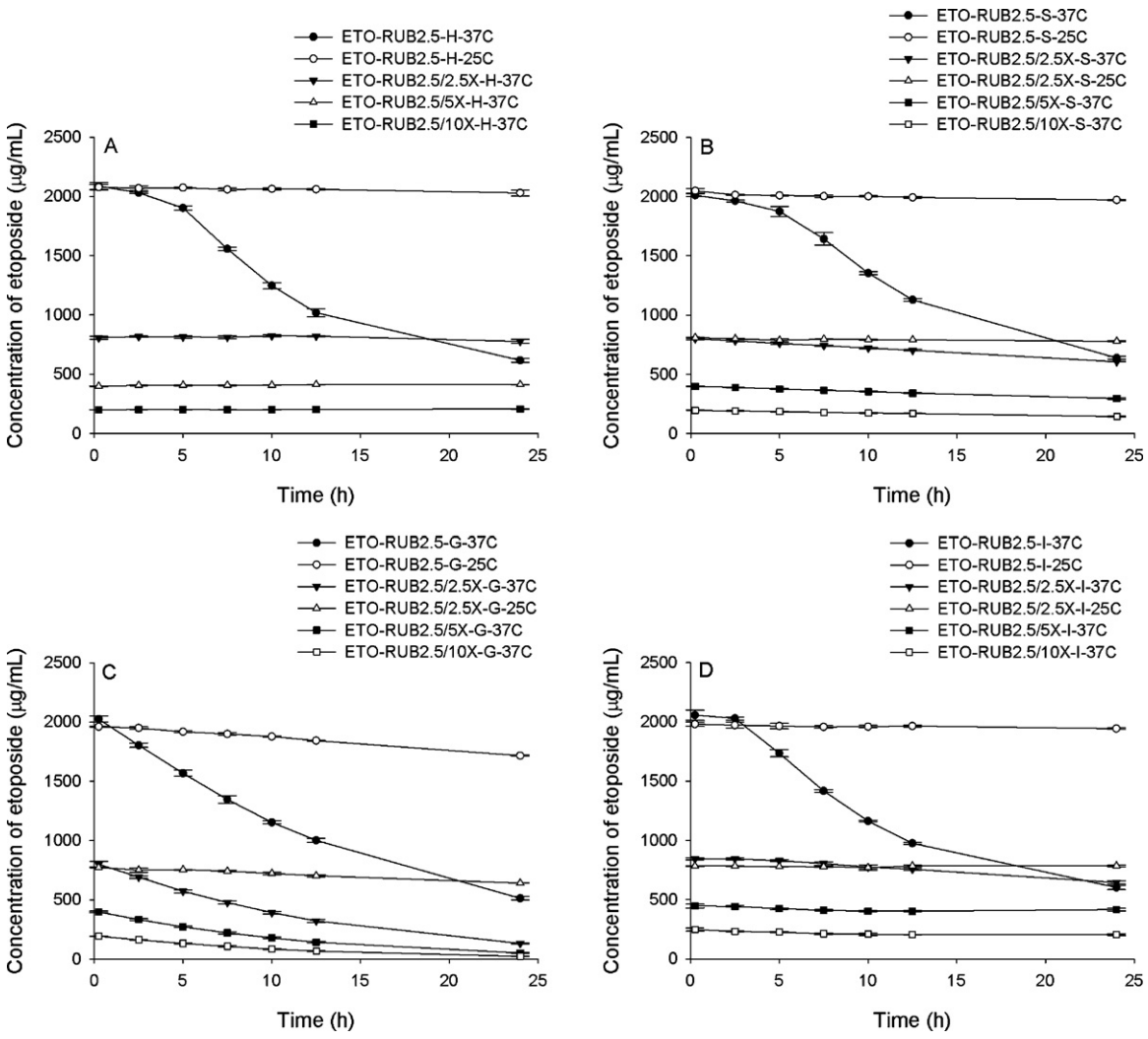

Fig. 3. Stability profiles of etoposide in physiological solutions over 24 h following ETO–RUB powder reconstitution and dilution by a factor of 2.5, 5 and 10 at 25 °C or 37 °C. (A) Water; (B) PBS solution; (C) simulated gastric fluid and (D) simulated intestinal fluid. Each data point represents the mean of three replicates. Vertical bars across each data point represent one standard deviation.

3.4. Cytotoxicity of the RUB-solubilized ETO

To evaluate the maintenance of cytotoxicity of RUB-solubilized ETO, a side-by-side comparison with DMSO-solubilized ETO was performed using a standard cell viability assay. Two identical ETO stock solutions (10.2 mM) were prepared either from absolute DMSO or by reconstituting the ETO–RUB powder in water to a final 0.1% (w/v) RUB. Stock solutions were diluted to an ETO range of 0.8–102.0 μM for the determination of IC_{50} values. ETO solubilized in RUB decreased the viability of HT-29, MDA-MB-231, and PC3 cancer cells in a concentration-dependent manner with IC_{50} values of 11.90 μM, 16.16 μM, and 54.46 μM, respectively (Table 2). RUB alone (vehicle) was found to have no effect on the viability of any cell line cultured at the highest ETO concentration (102 μM) used for this assay. ETO solubilized in DMSO was equally active against these three cell lines. ETO in DMSO-solubilized and RUB-solubilized formulations showed no significant differences in IC_{50} values ($P > 0.05$) in any of the tested cell lines.

4. Discussion

The low water solubility of etoposide (~ 30 μg/mL), due to its strong lipophilicity, requires the use of solubilizing excipients (IARC, 2000) for drug delivery. VePesid®, a widely prescribed brand of ETO, is available in two different formulations, each specific for a route of administration. The intravenous VePesid® is a clear yellow liquid containing 20 mg/mL ETO, which was formulated with multiple solubilizing and stabilizing excipients (2 mg/mL citric acid, 30 mg/mL benzyl alcohol, 80 mg/mL polysorbate 80, 650 mg/mL polyethylene glycol 300, and 0.305 mL/mL alcohol). The oral VePesid® drug is available in the form of a softgel capsule containing 50 mg of ETO in a vehicle consisting of citric acid, glycerin, purified water, and polyethylene glycol 400 (Drugs.com, 2011). Because of the side effects caused by the excipients in VePesid®, attempts of reformulation with other solubilizing agents have been

Table 2
Cytotoxicity expressed as mean IC_{50} ± standard error of etoposide solubilized in RUB or DMSO against three human cancer cell lines.

Cell Line	IC_{50} (μM)[a]	
	ETO in DMSO	ETO–RUB in H_2O
HT-29 (Colon)	8.42 ± 0.35 a[b]	11.90 ± 0.21 a
MDA-MB-231 (Breast)	8.69 ± 2.17 a	16.16 ± 0.99 a
PC3 (Prostate)	71.37 ± 3.9 a	54.46 ± 3.78 a

[a] Each value represents the average of two independent experiments.
[b] Same letters following each IC_{50} value in each cell line indicate no significant differences ($P > 0.05$) by the Student's *t*-test.

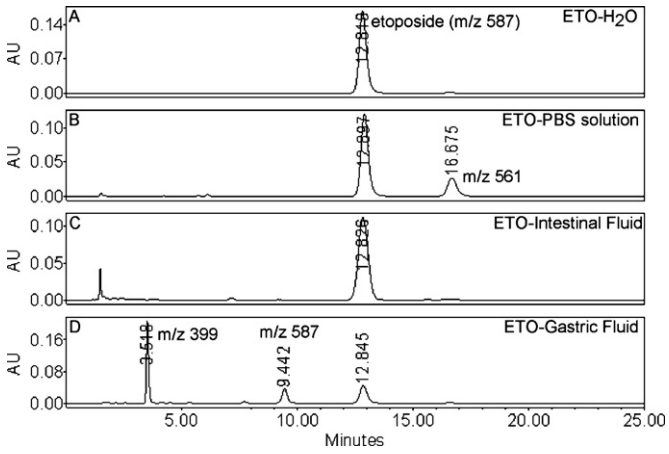

Fig. 4. HPLC chromatography at UV 285 nm of reconstituted ETO–RUB solutions kept at 37 °C for 20 h. (A) Water; (B) PBS solution; (C) simulated intestinal fluid and (D) simulated gastric fluid.

made. For examples, using PCL-PEG-PAMAM, stPCL-PEG32, or P-1-4 polymeric micelles, an ETO concentration of 0.95, 0.50, or 4.3 mg/mL in water was detected, respectively (Gaucher et al., 2007; Wang et al., 2008; Li et al., 2010). This study demonstrated a novel and more effective solubilization method. Furthermore, by using 200 mg/mL RUB singly, solubilization to a concentration of 20 mg/mL ETO in water was achieved (data not shown). Additionally, powdered ETO rather than liquid ETO enables the solid dosage form so that the current refrigeration requirement for VePesid® may become unnecessary. Perhaps most importantly, steviol glycosides are found to be safe for oral ingestion (WHO, 2006) and are in numerous US FDA-granted GRAS (generally regarded as safe) petitions as food sweetening agents (GRAS Notice Nos. 252, 253, 275, 278, 282, 287, 303, 304, 318, 323, 329, 348, 349, 354, 365, 367). Replacing the toxic excipients with RUB may significantly improve safety and reduce side effects.

Solubility and stability are two key aspects of successful formulations (Strickley, 2004). In addition to insolubility, the instability of ETO in the *i.v.* and oral formulations poses another challenge. ETO has a tendency to precipitate when being diluted for *i.v.* infusion. It was indicated that the VePesid® concentrate, when diluted to 1.0 mg/mL or higher, or to 0.4 mg/mL and administered through tubing connected to a peristaltic pump, may precipitate out of solution upon dilution or in the tubing (Hande, 1998). ETO in the RUB nanoparticles, in contrast, has shown superior physical stability in PBS solution that was free of precipitation over a 24-h period and capability of infusing a concentration of 1 mg/mL or higher ETO. In other studies ETO stability was found to be affected by pH, which may have important implications for oral administration (Shah et al., 1989). ETO was most stable at a pH of 5.26. At a lower pH, it has been shown to lose stability by both precipitation and degradation to its aglycone form. While in basic solutions, ETO epimerized to its *cis*-lactone leading to loss of the ETO structure (Beijnen et al., 1988; Tian et al., 2007). Simon etc. reported that, in simulated gastric fluid, about 40% of ETO with the starting concentration of 1.0 mg/mL got lost in 8 h. In simulated intestinal fluid, about 50% of ETO with the starting concentration of 1.0 mg/mL got lost in 8 h (Joel et al., 1995b). The physical and chemical instability of ETO in the gastric and intestinal fluids indicates that only approximately half of orally administered ETO reaches the intestinal absorption interface. By solubilizing ETO with RUB such reported losses were much lower (13% in simulated gastric fluid, 2% in simulated intestinal fluid). This improved stability should lead to more accurate delivery of intended ETO amount for intestinal absorption.

Although the exact mechanisms behind the apparent solubilization of ETO remain unclear, structural analyses of RUB supplemented with the FF-TEM and DLS data shed some light on the formation of water-soluble nanomicelles between RUB and ETO. Because RUB is an amphiphilic molecule, it is not unreasonable to suspect that RUB molecules can self-associate to minimize the exposure of their central groups to water, as in other bolaform amphiphiles (Claussen et al., 2003).

A side-by-side comparison of ETO stock solutions (10 mM) prepared in absolute DMSO or by RUB showed that the cytotoxic property of ETO was each maintained. This shows that ETO reformulated in RUB was bioavailable to the cells and efficacious, and warrants the next steps of toxicology, bioavailability, and efficacy validations in animal models.

5. Conclusions

A novel solubilization method for the poorly soluble etoposide by the use of rubusoside was demonstrated. This solubilization method shows a better solubilization effect and capability of improving physical and chemical stability profiles than the reported existing formulations. These features may improve bioavailability and clinical efficacy while simultaneously improving safety, benefiting from the GRAS status of rubusoside.

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