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Development, stability, and pharmacokinetics in Sprague-Dawley rats

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**High-dose etoposide formulations do not saturate intestinal P-glycoprotein: Development,  
stability, and pharmacokinetics in Sprague-Dawley rats**

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21 **Abstract:** It has been suggested that oral absorption of low-permeable P-glycoprotein (P-gp)  
22 substrates can be increased through saturation of P-gp. For BCS class IV drug substances, saturating  
23 P-gp is challenging due to low aqueous solubility. The present study investigated if the BCS IV drug  
24 substance etoposide could be solubilized to a concentration saturating P-gp after oral administration.  
25 A formulation consisting of 10% (w/v) of pluronic® F-127 and polyvinylpyrrolidone/vinyl acetate  
26 (PVP/VA), and 57% (v/v) ethanol enhanced etoposide's solubility approximately 100 times (16 mg  
27 mL<sup>-1</sup>) compared to its aqueous solubility. *In vitro*, this formulation was stable upon dilution in  
28 simulated intestinal fluid. In male Sprague-Dawley rats, oral administration of increasing solubilized  
29 etoposide doses using the formulation matrix increased the AUC<sub>0-∞</sub> of etoposide dose-proportionally  
30 but resulted in a lower absolute oral bioavailability (F) and rate of absorption as compared to control.  
31 At the highest investigated dose (100 mg kg<sup>-1</sup>), AUC<sub>0-∞</sub> and C<sub>max</sub> were significantly increased by 2.9-  
32 and 1.4-fold, respectively, compared to control dosed at 20 mg kg<sup>-1</sup>. A single oral dose of 20 mg kg<sup>-1</sup>  
33 zosuquidar followed by 20 mg kg<sup>-1</sup> oral etoposide increased F 8.6-fold. In conclusion, a stable  
34 formulation with improved etoposide solubility was developed, yet the formulation did not result in  
35 increased oral bioavailability of etoposide.

36

37 **Keywords:** P-glycoprotein, Etoposide, Zosuquidar, Population pharmacokinetics, Sprague-Dawley  
38 rats.

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## 43 1. Introduction:

44 P-glycoprotein (P-gp, MDR1) has been shown to limit the intestinal absorptive permeability of low-  
45 permeable substrates in cell cultures (Alsenz et al., 1998; Collett et al., 1999; Sparreboom et al., 1997;  
46 Terao et al., 1996; Troutman and Thakker, 2003b). *In vivo*, in wild type (WT) rats, P-gp restricts the  
47 oral bioavailability of several P-gp substrates such as loperamide (Zamek-Gliszczyński et al., 2012),  
48 paclitaxel (Zamek-Gliszczyński et al., 2012), digoxin (Nielsen et al., 2016), and etoposide (Al-Ali et  
49 al., 2018a). This is evident since the oral bioavailability is significantly increased in *mdr1a* deficient  
50 rats (Al-Ali et al., 2018a; Nielsen et al., 2016; Zamek-Gliszczyński et al., 2012). Moreover, co-  
51 administration of P-gp substrates with potent small-molecular P-gp inhibitors such as verapamil  
52 (Tsuruo et al., 1981), dexverapamil (Gramatté and Oertel, 1999), valspodar (PSC-833) (Mayer et al.,  
53 1997; vanAsperen et al., 1997), or zosuquidar (LY335979) (Bardelmeijer et al., 2004) increased the  
54 oral bioavailability of the substrates in preclinical studies. However, using inhibitors to increase the  
55 oral bioavailability of P-gp substrates in some cases also affects metabolism via the cytochrome  
56 P450s and results in adverse effects (Breedveld et al., 2006; Palmeira et al., 2012; Varma et al., 2003;  
57 Varma and Panchagnula, 2005). Other studies have shown that co-administration of pharmaceutical  
58 excipients such as nonionic surfactants may also have the potential to increase the oral bioavailability  
59 of digoxin (Cornaire et al., 2004; Nielsen et al., 2016; Zhang et al., 2003), etoposide (Akhtar et al.,  
60 2017; Al-Ali et al., 2018a), and paclitaxel (Varma and Panchagnula, 2005) in WT rats. To overcome  
61 the cellular efflux mediated by P-gp to increase intestinal absorption, it should in theory be possible  
62 to saturate the transporters by achieving intestinal concentrations well above the  $K_m$ -value for the  
63 substrate-transporter binding as mentioned by e.g. Lin and Yamazaki (Lin and Yamazaki, 2003).  
64 Taking into consideration the degree of passive diffusion, intestinal metabolism, and P-gp substrate  
65 binding affinity, this may be a feasible strategy for drug substances with sufficient aqueous solubility,  
66 low passive diffusion, and limited metabolism. The experimental evidence for the feasibility of

67 saturating P-gp in the intestine through a pharmaceutical formulation strategy is however relatively  
68 limited and ambiguous. Lin and Yamazaki and Chiou et al., retrospectively, interpreted the  
69 pharmacokinetic human studies of the BCS II compound talinolol made by de Mey et al., and  
70 concluded that since the dose normalized AUC values after oral administration of increasing talinolol  
71 doses increased with higher doses, this suggested saturation of P-gp efflux (Chiou et al., 2001; de  
72 Mey et al., 1995; Lin and Yamazaki, 2003). Talinolol is also a substrate of absorptive solute carriers  
73 including organic anion transporting polypeptide 1A2 (OATP1A2) and OATP2B1 (Shirasaka et al.,  
74 2010) making the absorption kinetics quite complicated.

75 Since high doses of a P-gp substrate are needed to saturate the transporter, and as most P-gp substrates  
76 are poorly water-soluble substances (Wang et al., 2001), suitable pharmaceutical formulation  
77 approaches are required to solubilize the substrate. SMEEDs formulations could be used, but they  
78 often contain surfactants that are also P-gp inhibitors see e.g. Zhao et al. (Zhao et al., 2013). Thus, in  
79 the present study a formulation that can maintain the substrate in a solubilized-form upon dilution in  
80 the intestinal lumen without P-gp inhibiting surfactants was developed using etoposide a BCS class  
81 IV P-gp substrate. Etoposide was selected since intestinal P-gp limited oral etoposide bioavailability  
82 in WT rats, whereas etoposide was completely absorbed in *mdr1a* deficient rats (Al-Ali et al., 2018a).  
83 Moreover, data suggests that Bcrp has very little impact on the intestinal absorption of etoposide in  
84 mice (Allen et al., 2003). A formulation was designed with ethanol and surfactant to enhance the  
85 solubility of etoposide. Etoposide has a low aqueous solubility of 0.15-0.2 mg mL<sup>-1</sup> (Beig et al., 2015;  
86 Darwish et al., 1989) and since 10% ethanol significantly enhanced etoposide stability in artificial  
87 intestinal fluid, ethanol was used as a solubilizing co-solvent for etoposide (Joel et al., 1995). Since  
88 many nonionic surfactants inhibit P-gp *in vitro* (Al-Ali et al., 2019; Al-Ali et al., 2018b; Batrakova  
89 et al., 1999; Batrakova et al., 2003; Gurjar et al., 2018; Li-Blatter et al., 2012; Lo, 2003; Zhao et al.,  
90 2016) and *in vivo* (Akhtar et al., 2017; Al-Ali et al., 2018a; Ma et al., 2011; Nielsen et al., 2016; Zhao

et al., 2013), a surfactant without P-gp inhibiting properties was chosen to evaluate the effect of increasing the dose of etoposide without a potential surfactant mediated P-gp inhibitory effect. Previous *in vitro* studies have reported that pluronic® F-127 did not inhibit the P-gp-mediated efflux of several substrates e.g. rhodamine 123 (Al-Ali et al., 2019; Batrakova et al., 2003; Wei et al., 2013), nelfinavir (Shaik et al., 2008), digoxin (Gurjar et al., 2018), and etoposide (Al-Ali et al., 2018a), hence this surfactant was chosen. Polyvinylpyrrolidone-vinyl acetate (PVP/VA) was suggested as a potential precipitation inhibitor (Kalaiselvan et al., 2006; Knopp et al., 2016; Xu and Dai, 2013) and formulation stabilizer during storage (Knopp et al., 2016; Prudic et al., 2014), thus it was included in the formulation.

The aim of the present study was to prepare an oral formulation of high concentration of etoposide and to investigate the stability on storage, and upon dilution in fasted state simulated intestinal fluid (FaSSIF). Furthermore, the formulation was used to investigate if the systemic exposure of orally administered etoposide in rats could be increased through saturation of P-gp transport activity.

104

## 2. Material and methods:

### 2.1. Materials

Caco-2 cells were obtained from the American Type Culture Collection (ATCC). Cell culture media and Hanks balanced salt solution (HBSS) were obtained from Life Technologies (Høje-Taastrup, Denmark). Etoposide (>99% purity) was purchased from Selleckchem (Munich, Germany). Sodium chloride, sodium taurocholate, sodium hydroxide, potassium chloride, L- $\alpha$ -phosphatidylcholine (approx. 60%), potassium phosphate monobasic ( $\geq 97\%$ ), and pluronic® F-127 suitable for cell culture, 2-(N-morpholino) ethane sulfonic acid (MES), N-[2-Hydroxyethyl] piperazine N'-[2-ethanesulfonate] (HEPES), verapamil, digoxin, and bovine serum albumin (BSA) were from Sigma

114 (Brøndby, Denmark). Ethanol (99.5 %) was from VWR Chemicals (Fontenay-sous-Bois, France).  
115 Polyvinylpyrrolidone-vinyl acetate (Kollidon<sup>®</sup> VA 64, referred as PVP/VA) meets Ph. Eur. standards  
116 and was from BASF SE (Ludwigshafen, Germany). Transwell<sup>®</sup> inserts with polycarbonate membrane  
117 (0.4  $\mu\text{m}$  pore size, 1.12  $\text{cm}^2$ ) were from Corning Life Sciences and purchased through Sigma Aldrich  
118 (Brøndby, Denmark). Whatman<sup>®</sup> Nuclepore<sup>™</sup> Track-Etched Membranes (0.03  $\mu\text{m}$  pore-size, 19 mm  
119 in diameter) were purchased from Sigma Aldrich (Brøndby, Denmark). LOCTITE<sup>®</sup> 401 glue was  
120 purchased from RS Components Ltd. (Corby, UK). <sup>3</sup>H-etoposide (specific activity 700 mCi mmol<sup>-1</sup>)  
121 was from Moravek (CA, USA). <sup>14</sup>C-glycine (specific activity 87 mCi mmol<sup>-1</sup>) was purchased from  
122 Larodan (Solna, Sweden) and <sup>14</sup>C-mannitol with a specific activity of 51.50 mCi/mmol was from  
123 Sigma Aldrich (Brøndby, Denmark). Ultima Gold liquid was purchased from Perkin Elmer  
124 (Skovlunde, Denmark). The radio-chemical purity of the isotopes was greater than 97%.

125

## 126 2.2. Cell culture and transport experiments

127 Caco-2 cells were cultured as previously described by Nielsen and co-workers (Nielsen et al., 2001).  
128 Briefly, cells were seeded in culture flasks and passaged in Dulbecco's Modified Eagle's medium  
129 (DMEM) supplemented with 10 % fetal bovine serum, penicillin/streptomycin (100 U ml<sup>-1</sup> and 100  
130  $\mu\text{g ml}^{-1}$ , respectively), 1 % L-glutamine, and 1 % non-essential amino acids. Cells were seeded onto  
131 tissue culture treated Transwells (1.0  $\text{cm}^2$ , 0.4  $\mu\text{m}$  pore size) at a density of 10<sup>5</sup> cells  $\text{cm}^{-2}$ .  
132 Transepithelial electrical resistance (TEER) at room temperature was measured before the  
133 experiment. All TEER values of monolayers used were >400  $\Omega \text{ cm}^2$ . Transport experiments were  
134 performed on day 24-28 after seeding.

135 Apical (A) to basolateral (B) and B to A fluxes of tritium labeled etoposide was measured in 10 mM  
136 HEPES buffers adjusted to pH 7.4. The concentration of etoposide on the donor (cis) side was 20 –

137 520  $\mu\text{M}$ . Samples (20  $\mu\text{l}$ ) were taken from the donor solution at  $t=0$  and 210 min and 150  $\mu\text{l}$  samples  
138 were taken from the receiver solution and replaced with fresh buffer ( $t=30, 60, 90, 120, 150, 180$ , and  
139 210 min). The transport of  $^3\text{H}$ -etoposide was also measured in the presence of 100  $\mu\text{M}$  of digoxin and  
140 50  $\mu\text{M}$  verapamil on both donor and receiver side, respectively. Samples were transferred to  
141 scintillation vials, where 2 ml of scintillation fluid was added and the radioactivity was counted in a  
142 liquid scintillation analyzer. Fluxes were constant after 60 min. The steady state flux values were thus  
143 obtained as the means of the flux values between 90-210 min. After the experiment, the integrity of  
144 the Caco-2 cell monolayers was evaluated by  $^{14}\text{C}$ -mannitol transport studies. Samples were taken  
145 from the donor chamber (10  $\mu\text{l}$ ) at 20, 40, and 60 min, and from the receiver chamber at 0, 20, 40,  
146 and 60 min. The permeability ( $P_{\text{app}}$ ) of mannitol was unaffected by etoposide, digoxin and verapamil  
147 and had a  $P_{\text{app}}$  value of  $1.4 \cdot 10^{-06} \pm 4.2 \cdot 10^{-07} \text{ cm s}^{-1}$ . The transport across the filter without Caco-2 cells  
148 was investigated in order to assess whether the filter was a barrier to diffusion of etoposide. The  
149 permeability across the filter was calculated using non-steady state kinetics and had a value of  $3.1 \cdot 10^{-04} \pm 4.2 \cdot 10^{-05} \text{ cm s}^{-1}$ .

151 The P-gp dependent flux ( $J_{\text{P-gp}}$ ) was calculated similarly to described by Troutman and Thakker  
152 (Troutman and Thakker, 2003a), where the total flux of etoposide was subtracted with the flux of  
153 etoposide in the presence of 100  $\mu\text{M}$  of digoxin and 50  $\mu\text{M}$  verapamil to yield the concentration  
154 dependent effect of P-gp on etoposide transport. Here we used Caco-2 cells from ATCC, and  
155 proteomics data suggest that BCRP expression is very low in the substrain compared to Caco-2 cells  
156 obtained from The European Collection of Authenticated Cell Cultures (ECACC) or/and Deutsche  
157 Sammlung von Mikroorganismen und Zellkulturen (DSMZ), as reviewed in Al-Ali et al., 2019 (Al-  
158 Ali et al., 2019). In Caco-2 cells from DSMZ it has been shown that 25  $\mu\text{M}$  zosuquidar abolish  
159 polarized etoposide transport completely (Nielsen et al., 2020), while it has also been shown that



160 verapamil completely abolishes polarized etoposide in Caco-2 cells (Mo et al., 2011). Therefore, it  
161 seems likely that in Caco-2 cells from ATCC BCRP has a minor impact on etoposide transport.  
162 The resulting etoposide flux in the B-A direction was then fitted to the following equation:

163

164 
$$J_{P-gp} = \frac{J_{P-gp,max} * C_d}{K_{m,app} + C_d} \quad (1)$$

165 where,  $J_{P-gp}$  is the P-gp mediated flux,  $J_{P-gp,max}$  is the maximal flux,  $K_{m,app}$  is the apparent Michaelis  
166 constant and  $C_d$  is the donor concentration.

167

### 168 2.3. Animals

169 *In vivo* pharmacokinetic studies were performed in WT male Sprague-Dawley rats, which were from  
170 Charles River Laboratories (Sulzfeld, Germany). The study protocol was approved by the local ethical  
171 committee in accordance with EC Directive 2010/63/EU and Belgian Law/1991 for Protection of  
172 Vertebrate Animals used for Experimental and other Scientific Purposes. The rats were received one  
173 week prior to the *in vivo* experiments, acclimatized and maintained on standard feed conditions with  
174 free access to water. The animals were fasted for 16 h before the pharmacokinetic studies. During the  
175 experiments, the rats had free access to water, but not food. At the day of the experiment, the weight  
176 of the animals was 242-275 g.

177

### 178 2.4. Preparation of etoposide-containing formulations

179 Different formulations of etoposide were prepared using the nonionic surfactant pluronic® F-127, the  
180 copolymer PVP/VA, ethanol, and ultra-purified water (Table 1). Pluronic® F-127 was first dissolved  
181 in 57% (v/v) ethanol solution at room temperature. Etoposide was then added to the surfactant-

182 containing solution and placed in a water bath at 40° C for 15-30 min, before the solution was mixed  
183 using a magnetic stirrer for 15-30 min. The solution was visually inspected for undissolved particles.  
184 PVP/VA powder was subsequently added, and the mixture placed in a water bath at 37° C for 15-30  
185 min, before being mixed using a magnetic stirrer for 15-30 min. The solution was again visually  
186 investigated for undissolved particles. Finally, provided that the etoposide-containing formulation  
187 was clear, the formulation was transferred to a glass container, protected from light, and kept at room  
188 temperature overnight (approx. 15 h). If no sign of precipitation appeared after 15 h, the formulation  
189 was further investigated for precipitation upon dilution with FaSSIF, see details below.

190

#### 191 *2.5. Monitoring of etoposide containing-formulations diluted with fasted state simulated* 192 *intestinal fluid*

193 Monitoring that etoposide does not precipitate in the different formulations when diluted with FaSSIF  
194 was investigated as previously described by Al-Ali et al. (Al-Ali et al., 2018a). Briefly, FaSSIF was  
195 prepared according to Galia et al., composed of 0.39 g potassium phosphate monobasic, 0.77 g  
196 potassium chloride, 3 mM sodium taurocholate, 0.75 mM L- $\alpha$ -phosphatidylcholine, and ultra-purified  
197 water added to 100 mL (Galia et al., 1998). The pH was then adjusted to  $6.50 \pm 0.05$  with sodium  
198 hydroxide before the osmolality was adjusted to  $270 \pm 10$  mOsm kg<sup>-1</sup> using sodium chloride (Galia  
199 et al., 1998). The different formulations of etoposide were then added to FaSSIF in a ratio of 1:2  
200 (formulation:FaSSIF) to simulate the likely intestinal dilution in the rats (McConnell et al., 2008) and  
201 monitored for two hours by measuring the absorbance at 400 nm and 25° C every 10 min using a UV-  
202 spectrophotometer (Genesys 10-S from Thermo Fisher Scientific (WI, USA)). Before each  
203 measurement, solutions were mixed for 10 seconds using pipetting.

204

## 2.6. Dialysis studies investigating etoposide release from different formulations

Dialysis studies were designed and optimized as previously described by Al-Ali et al. (Al-Ali et al., 2018a), with minor modifications. In brief, the dialysis setup allows the diffusion of free-fraction of etoposide (unbound to micelles) and retains etoposide-bound to micelles, which should be not diffused across a 0.03  $\mu\text{m}$  pore-size polycarbonate membrane during the course of the experiment (2 h). Polycarbonate membranes (0.03  $\mu\text{m}$  pore size, 19 mm in diameter) were attached to Transwell holders (1.12  $\text{cm}^2$ ) by LOCTITE® 401 glue and left to dry at room temperature overnight (Al-Ali et al., 2018a). The Transwell inserts, with attached polycarbonate membranes, were then hydrated overnight in ultra-purified water. On the day of the experiment, Transwell inserts with polycarbonate membranes were first pre-incubated with HBSS supplemented with 10 mM HEPES and adjusted to pH  $7.40 \pm 0.05$  (HBSS<sup>+</sup>) on a shaking plate at 220 rpm at 37° C for 10 min. Donor solutions of <sup>3</sup>H-etoposide and <sup>14</sup>C-glycine were prepared in HBSS<sup>+</sup> (Control), or in different formulation-matrices containing 10% (w/v) PVP/VA, 57% (v/v) ethanol, and pluronic® F-127 at 1.5, 3 or 10%, referred to as Matrix-PF127 1.5%, Matrix-PF127 3%, or Matrix-PF127 10%, respectively, or mixed with a formulation with the highest concentration of etoposide. This formulation contained 15-16  $\text{mg mL}^{-1}$  etoposide, 10% (w/v) of pluronic® F-127 and PVP/VA, and 57% (v/v) ethanol. The solution containing radioactive compounds was then added to FaSSIF at a 1:2 ratio to achieve final concentrations of <sup>3</sup>H-etoposide and <sup>14</sup>C-glycine at 0.25  $\mu\text{Ci mL}^{-1}$ , 0.36  $\mu\text{M}$  and 0.25  $\mu\text{Ci mL}^{-1}$ , 2.87  $\mu\text{M}$ , respectively, before these isotopes-containing solutions (500  $\mu\text{L}$ ) were added to the upper side of the polycarbonate membrane. Experiments started when receiver-release media (1650  $\mu\text{L}$ ) was loaded in the lower side of the polycarbonate membrane. Receiver-release media consisted of HBSS buffer supplemented with 10 mM HEPES and adjusted to pH  $6.50 \pm 0.05$  for all conditions except for the formulation containing the highest concentration of etoposide where the medium was the matrix of this formulation (i.e. 10% of pluronic® F-127 and PVP/VA, and 57% ethanol without

etoposide) in order to prevent etoposide precipitation when accumulated in the receiver chamber. Experiments were performed at 37° C and shaking mode 220 rpm. Donor samples were collected at 0, 60 and 120 min, while receiver samples were collected at 15, 30, 60, 90, and 120 min. Receiver-release medium was replaced at each sampling occasion in order to preserve the sink condition. Finally, Ultima Gold scintillation fluid (2 mL) was added to every sample, vortexed for one minute and counted for 10 min on a scintillation counter (TriCarb 4910TR) from Perkin Elmer, USA.

## 2.7. Pharmacokinetic study in wild-type male Sprague-Dawley rats

The rats were randomly assigned to receive different doses of etoposide in ethanol solution or in a formulation matrix consisting of 10% (w/v) pluronic® F-127 and PVP/VA, 57% (v/v) ethanol, and ultra-purified water. For intravenous administration, the rats received etoposide solution (2.86 mg mL<sup>-1</sup> in 57% v/v ethanol) at a dose of 5 mg kg<sup>-1</sup> etoposide. Administration volume was adjusted to 2 mL with water before dosing the rats. For oral administration by oral gavage, the control group received an etoposide solution (2.86 mg mL<sup>-1</sup> in 57% v/v ethanol) at a dose of 20 mg kg<sup>-1</sup> etoposide and a dosing volume of 7 mL kg<sup>-1</sup>. In the positive control group, the rats received a zosuquidar solution (2 mg mL<sup>-1</sup>) at a dose of 20 mg kg<sup>-1</sup> and dosing volume of 10 mL kg<sup>-1</sup>, 30 min before the administration of etoposide doses which were administered in a similar fashion to the control group. For oral administration of increasing doses of etoposide, an increasing concentration of etoposide was solubilized in the formulation matrix i.e. 2.86, 7.15 and 14.3 mg mL<sup>-1</sup>. These etoposide-containing formulations were administered at doses of 20, 50, and 100 mg kg<sup>-1</sup>, respectively, in a dosing volume of 7 mL kg<sup>-1</sup>. Each dosing group consisted of 5-6 male Sprague-Dawley rats. After oral or intravenous administration of etoposide, blood samples were collected at 0.25, 0.5, 0.75, 1, 2, 3, 4, and 6 h, as well as at 5 min (0.0833 h) post intravenous administration of etoposide. The samples were obtained

252 at each sampling point by puncturing the lateral tail vein using a 23G needle and approximately 35  
253  $\mu$ L of blood were collected using a capillary tube from VITREX Medical A/S (Herlev, Denmark).  
254 The capillaries with samples were then sealed with VITREX Sigillum Wax. Plasma samples were  
255 harvested immediately from these capillaries after centrifugation at 1900xg for 10 min at 4° C and  
256 transferred to a 10  $\mu$ L end-to-end capillary (VITREX) followed by storage at -20° C until further  
257 bioanalysis. At 6 h, the last samples were collected, and the animals were euthanized.

258

## 259 2.8. *Quantification of etoposide in pharmacokinetic studies*

260 Quantification of etoposide in plasma samples was performed on an API 4000™ LC/MSMS System  
261 from AB SCIEX (Ontario, Canada). Briefly, plasma samples were precipitated with acetonitrile after  
262 wash-out from the end-to-end capillaries. Chromatographic separation was carried out using an  
263 Acquity UPLC BEH C18 column (1.7  $\mu$ m, 2.1 mm  $\times$  50 mm). Gradient elution was performed at 50  
264 °C with 0.01M ammonium acetate (mobile phase A) and acetonitrile (mobile phase B). Starting  
265 conditions were 5 % B for 0.25 minutes, then a linear gradient to 95% B was applied over 1.25  
266 minutes, followed by an isocratic hold at 95% B for 0.5 min before re-equilibration at 5% B. Total  
267 run time was 2.5 min and a flow rate of 0.6 mL/min was applied.

268 The LC-MS/MS was operated in positive ion mode using the TurboIonSpray™-interface  
269 (electrospray ionization), and was optimized for the quantification of etoposide, applying multiple  
270 reaction monitoring ( $m/z$  587.2  $\rightarrow$   $m/z$  381).

271 Incurred samples were quantified against calibration curves prepared in blank rat plasma. Independent  
272 quality control samples were included to evaluate accuracy (between 80 and 120% of the nominal

value) of the analytical run. The concentrations were correlated linearly, with a  $1/x^2$  weighing, with the MS response between 1.00 and 10000 ng mL<sup>-1</sup>.

275

## 2.9. Population Pharmacokinetic analysis

Population pharmacokinetic analysis was performed using Monolix 2018R2 (Lixoft SAS, Antony, France) based on a two-compartment population pharmacokinetic model with linear first order elimination and an oral depot compartment with subsequent first order absorption. Population pharmacokinetic parameter estimates were generated using the stochastic approximation expectation-maximization (SAEM) algorithm. Modeling was performed using the clearance parameterization assuming a lognormal distribution of individual parameters. The individual parameter estimates for the  $i$ th subject were modeled according to equation 2.

$$\theta_i = \theta_{pop} \cdot e^{\eta_{\theta,i}} \quad (2)$$

where  $\theta_i$  is the individual parameter estimate for the  $i$ th subject,  $\theta_{pop}$  is the typical population parameter estimate and  $\eta_{\theta,i}$  is assumed to be the random individual deviation for the  $i$ th subject for a particular population parameter  $\theta$ . The clearance parametrization included the volume of the central compartment ( $V_1$ ), the elimination clearance (CL), the volume of the peripheral compartment ( $V_2$ ), and the inter-compartmental flow between the central and the peripheral compartment ( $Q_2$ ). Oral administration was modeled using a dosing compartment and subsequent first order absorption. The final model included covariate effects, which partly explained differences observed between animals assigned to the different formulation groups. Forward inclusion of covariates was judged based on the decrease in objective function value expressed as -2 log likelihood (-2LL) and Akaike's information criterion (AIC). Covariate selection was guided by physiological plausibility and

295 statistical significance ( $P < 0.05$ ). Covariate effects of the final population model included the  
 296 presence of zosuquidar on F and the presence of experimental matrix (referred as formulation G-  
 297 matrix) on F and  $k_a$ . The final model estimated inter-individual variability for F,  $k_a$ ,  $V_1$  and CL.  
 298 Residual error was accounted for by a combined (additive and proportional) error model (Equation  
 299 3).

$$300 \quad \log(Y_{ij}) = \log(c_{\text{pred},ij}) + (a + b \cdot \log(c_{\text{pred},ij}))\epsilon_{ij} \quad (3)$$

301 where the  $j$ th observation of the  $i$ th subject deviates from the  $j$ th prediction of the  $i$ th subject,  $c_{\text{pred},ij}$ ,  
 302 by an additive term,  $a$ , and a proportional term,  $b$ .  $\epsilon_{ij}$  was assumed to be the random variable for the  
 303  $j$ th concentration of the  $i$ th individual, sampled from a distribution with zero as a mean and a variance  
 304 of  $\sigma^2$ . Individual Bayesian estimates were used to calculate mean  $\text{AUC}_{0-\infty}$  per study cohort. Individual  
 305  $\text{AUC}_{0-\infty}$  values ( $\text{AUC}_{0-\infty,i}$ ) were calculated as shown in equation 4.

$$306 \quad \text{AUC}_{0-\infty,i} = \frac{F_i \cdot D}{\text{CL}_i} \quad (4)$$

307

## 308 2.10. Statistics

309 The results of *in vitro* studies were obtained from at least three experiments and presented as mean  
 310 values  $\pm$  SEM. Caco-2 cell experiments were conducted in three independent cell passages and values  
 311 are presented as mean values  $\pm$  SEM. The results of the *in vivo* pharmacokinetic studies were obtained  
 312 from 5 or 6 rats per study cohort and data are presented as mean values  $\pm$  SEM. Statistical analysis  
 313 was performed using GraphPad Prism 7.01 from GraphPad Software, LLC (San Diego, CA, USA).  
 314 One-way ANOVA test followed by Tukey's multiple comparisons test were selected for multiple  
 315 comparisons. P value  $< 0.05$  was considered statistically significantly different from control.

Moreover, dose-proportionality has been assessed by plotting  $AUC_{0-\infty}$  as a function of oral etoposide doses and linear regression analysis was performed.

318

### 3. Results

#### 3.1. Transepithelial flux of etoposide across Caco-2 cells

Etoposide transport across Caco-2 monolayers was investigated, and in the following Figure 1, the A-B and B-A flux dependent on P-gp is shown. The A-B flux dependent on P-gp shows a gradual increase as a function of concentration and at higher concentrations the flux is relatively linear as a function of concentration in the apical donor solution (Fig. 1A). In the B-A direction, the flux of etoposide across Caco-2 cells monolayers can be described by Michaelis-Menten like kinetics where the flux becomes saturated with increasing concentration in the basolateral medium. The resulting kinetical parameters were estimated at  $257 \pm 32 \mu\text{M}$ ,  $199 \pm 17 \text{ pmol cm}^{-2} \text{ min}^{-1}$ , and  $1.7 \pm 0.2$  for  $K_{m,app}$ ,  $J_{P-gp, max}$ , and the Hill coefficient, respectively.

329

#### 3.2. Etoposide did not precipitate in formulations containing high percentages of ethanol

Different formulations of etoposide were prepared using 44% or 57% (v/v) ethanol, 2-10% (w/v) pluronic<sup>®</sup> F-127, and 0-15% (w/v) PVP/VA (Table 1). Etoposide in formulations containing 44% (v/v) ethanol either contained low concentration of etoposide such as  $9 \text{ mg mL}^{-1}$  (Formulation A), which was not sufficient to perform our study or precipitated after overnight incubation such as Formulation B (Fig. 2). Etoposide formulations (C-G) showed no signs of etoposide precipitation after overnight incubation; e.g. Formulation G as shown in Figure 2. Etoposide solubility in these formulations containing 57% (v/v) ethanol was higher than in formulation A and B. Formulation D showed that 6% (w/v) of the surfactant was required to dissolve  $15 \text{ mg mL}^{-1}$  etoposide in PVP/VA-



339 free formulation. Formulations A, C, and E-G were designed to contain 5-15% (w/v) PVP/VA as a  
340 potential precipitation inhibitor. Formulation G consisting of 10% (w/v) of pluronic® F-127 and  
341 PVP/VA, 57% ethanol, and water could solubilize an amount of etoposide corresponding to a 100-  
342 fold increase compared to the aqueous solubility. Further studies were conducted to investigate  
343 whether etoposide precipitates after the addition of different formulations to FaSSIF.

344

345 *3.3. Etoposide did not precipitate from Formulation G after dilution in fasted state simulated*  
346 *intestinal fluid*

347 Formulations C-E showed a fast etoposide precipitation (less than 30 min) after dilution in FaSSIF.  
348 Formulation F started to precipitate after approx. one hour and reached similar levels of absorbance  
349 values as C-E at 90 min. Therefore, formulations C-F were only prepared and investigated once.  
350 Formulation G showed no sign of etoposide precipitation for 120 min (Fig. 3). After 120 min,  
351 etoposide started to precipitate indicating that the matrix of formulation G was able to maintain  
352 etoposide in a solubilized form in FaSSIF for two hours (Fig. 3), which is the period that likely covers  
353 the absorption phase of etoposide in rats. Equal amounts of pluronic® F-127 and PVP/VA at 10%  
354 (w/v) were required to maintain etoposide in a solubilized form when diluted with FaSSIF. Among  
355 the investigated formulations, formulation G was stable in FaSSIF and the next step was to investigate  
356 if the formulation G would retain etoposide.

357

358 *3.4. Etoposide release was similar from different surfactant-containing solutions and matrix of*  
359 *formulation G*

360 As shown in Figure 4, simultaneous etoposide and glycine release from different pluronic® F-127-  
361 containing solutions and formulation G to receiver-release media increased time dependently. The  
362 release of etoposide and glycine from different pluronic® F-127-containing formulations and from  
363 formulation G was similar to the release in the control buffer i.e. without surfactant. Moreover, there  
364 was no statistically significant difference between the slopes of the time-dependent release of  
365 etoposide or glycine compared to control. The presence of non-radiolabeled etoposide in formulation  
366 G did not affect the release of radiolabeled etoposide during the course of the experiment.

367

368 *3.5. Etoposide population pharmacokinetics in WT male Sprague-Dawley rats after intravenous*  
369 *and oral administration*

370 Pharmacokinetic data available included 321 concentration-time points collected from 35 male WT  
371 Sprague-Dawley rats. The PK profile af intravenous administration of etoposide is shown in figure 5,  
372 illustrating a fast distribution phase followed by a slower elimination phase. Data points at 4 h post  
373 intravenous administration of etoposide are not shown in Figure 5 as the plasma concentration values  
374 were below the limit of quantification (LOQ) at this time point, and only three plasma-concentrations  
375 had values above LOQ at 6 h. Etoposide pharmacokinetic profiles after oral administration of  
376 etoposide-containing solutions were best described by a two-compartment structural model with  
377 linear elimination and first-order absorption (Fig. 6). Subsequently, a covariate model was developed  
378 to account for the inter-individual variability arising from the experimental design. The introduction  
379 of zosuquidar dose as a covariate on the oral bioavailability F, and matrix-G presence as a covariate  
380 on F as well as on the absorption rate constant ( $k_a$ ) significantly improved the model fit. After  
381 covariate inclusion, the unexplained inter-individual variability for F and  $k_a$ , calculated as the square  
382 root of the exponential variance of  $\eta$  minus 1, decreased from 153% for F and 59.7% for  $k_a$  to 15.9%

383 and 53.5%, respectively, from the base structural model to the final covariate model ( $\Delta 138\%$  for F  
384 and  $\Delta 6.15\%$  for  $k_a$ ). Estimated population pharmacokinetic parameters, precision of the parameter  
385 estimates and objective function values of both the base structural model and the final covariate model  
386 are presented in Table 2. Empirical Bayesian estimates were used to calculate  $AUC_{0-\infty}$  and dose  
387 normalized  $AUC_{0-\infty}$ .  $AUC_{0-\infty}$ , dose normalized  $AUC_{0-\infty}$ ,  $t_{1/2}$ ,  $t_{max}$  and  $C_{max}$  are presented in Table 3.  
388 Rats pretreated with zosuquidar showed the largest etoposide exposure ( $AUC_{0-\infty}$  of  $4511 \text{ ng h mL}^{-1}$   
389  $\text{kg}$ ). Dose-proportional etoposide exposure was observed for the 20, 50, and  $100 \text{ mg kg}^{-1}$  dosing  
390 cohorts receiving etoposide prepared in the matrix of formulation G (i.e. 10% of pluronic® F-127 and  
391 PVP/VA, 57% ethanol, and water), and R square was equal to 0.99 when the AUC was plotted as a  
392 function of dose (supplementary figure S1). However, the F,  $k_a$ ,  $AUC_{0-\infty}$ , and  $C_{max}$  were significantly  
393 lower when the rats were dosed with etoposide  $20 \text{ mg kg}^{-1}$  dissolved in the matrix of formulation G  
394 compared to the control group. Etoposide oral bioavailability significantly increased when the rats  
395 were pretreated with zosuquidar. The oral pharmacokinetic profile showed that the oral absorption of  
396 etoposide was slower in matrix-based formulations as was also apparent from the decreased  $k_a$  values.

397

#### 398 4. Discussion

399 In the present study, a stable formulation containing high concentration of etoposide and ethanol  
400 (57%) was developed using a surfactant without P-gp inhibiting properties. After oral administration  
401 of etoposide in this formulation, no increased bioavailability related to P-gp saturation could be  
402 shown, illustrating the practical difficulties in saturating P-gp mediated transport of low soluble drug  
403 substances for increasing the oral absorption through a formulation approach.

404 To investigate if the transport of etoposide was saturable, we used the approach described by  
405 Troutman and Thakker (Troutman and Thakker, 2003a), in which the net flux due to P-gp mediated

transport is estimated by subtracting the flux under P-gp inhibited conditions. We found that the B-A transport was saturable with kinetical parameters similar to the ones described by Troutman and Thakker in Caco-2 cells, for  $K_{m,app}$  257 vs. 461  $\mu\text{M}$ , and for  $J_{max}$  199 vs. 354  $\text{pmol cm}^{-2} \text{min}^{-1}$  (Troutman and Thakker, 2003a). Makhey et al. found a similar  $K_m$  value of 113  $\mu\text{M}$  in Caco-2 cells and 94-119  $\mu\text{M}$  in stripped rat intestinal tissue (Makhey et al., 1998). Troutman and Thakker estimated a higher  $K_m$  value of 1360  $\mu\text{M}$  for the A-B transport direction (Troutman and Thakker, 2003a), however this value was outside the concentration range that could be investigated in our set-up. Even though the B-A transport appears saturable and while it is more difficult to be assessed from the A-B transport, studies in wild-type and knock-out rats clearly show that the apically located P-gp is highly attenuating the oral etoposide absorption (Al-Ali et al., 2018a). Considering the above mentioned  $K_m$  values, generally of approximately 100-500  $\mu\text{M}$ , an intestinal concentration of 10 times these values would likely be required to fully saturate P-gp mediated transport by etoposide itself, corresponding to approximately 5000  $\mu\text{M}$  for the worst case scenario. A solubility higher than that could be achieved with formulation G (16  $\text{mg mL}^{-1}$  (27183  $\mu\text{M}$ )) containing 10% of pluronic<sup>®</sup> F-127 and 10% PVP/VA. 16  $\text{mg mL}^{-1}$  is approximately 100-fold higher than the aqueous solubility of etoposide (Beig et al., 2015; Darwish et al., 1989). Pluronic<sup>®</sup> F-127 forms micelles that have the capacity to solubilize lipophilic drug substances e.g. meso-tetraphenyl porphine (mTPP) (Sezgin et al., 2007), rofecoxib (Ahuja et al., 2007), ibuprofen (Wan et al., 2010), paclitaxel, and lapatinib (Kelishady et al., 2015). The reported critical micelle concentration (CMC) values of pluronic<sup>®</sup> F-127 were 1, 0.1, and 0.025% (w/v) at 25, 30, and 35°C (Alexandridis and Hatton, 1995), respectively. Etoposide in micelles could be retained from permeation and hence the oral absorption could be decreased, which has been reported with 25% polysorbate 20 (Al-Ali et al., 2018a). However, the presence of different concentrations of pluronic<sup>®</sup> F-127 did not affect the *in vitro* release of etoposide and glycine compared to the release from a surfactant-free formulation. Similarly, 1% (w/v) pluronic<sup>®</sup>

430 F-127, which is above the CMC value, did not affect the release of the lipophilic drug rofecoxib  
431 (Ahuja et al., 2007) (Log P 2.56) (Chemicalize, 2018).

432 Previous pharmacokinetic studies in rats have shown that  $t_{\max}$  of etoposide exposure was between  
433 0.25-1.75 h (Al-Ali et al., 2018a; Li et al., 2009; Zhao et al., 2013), therefore the formulation should  
434 maintain etoposide in its solubilized form for two hours when diluted in FaSSIF. This period was  
435 considered sufficient for further *in vivo* studies. In formulation G, solubilized etoposide did not  
436 precipitate for two hours when diluted with FaSSIF. Thus, pluronic® F-127 probably had two  
437 functions: i) enhanced the solubility of etoposide, and ii) decreased the precipitation rate of etoposide  
438 as it was also suggested by (Li et al., 2012; Xu and Dai, 2013), thereby stabilizing the formulation in  
439 FaSSIF. Importantly, it seems that a balance between pluronic® F-127 and PVP/VA concentrations  
440 (i.e. 10% of each) in the formulation was needed to achieve these formulation characteristics. Thus,  
441 if an intestinal dilution of 3-10 is expected after oral administration of a 16 mg mL<sup>-1</sup> etoposide  
442 formulation, and initial estimated intestinal concentration of 2718 – 9061 µM could be obtained,  
443 which could be able to saturate P-gp assuming that etoposide remains in solution.

444 In rats receiving 100 mg kg<sup>-1</sup> of etoposide in formulation G, a higher AUC<sub>0-∞</sub> was obtained compared  
445 to rats receiving 20 mg kg<sup>-1</sup> etoposide in a 57% ethanol solution. Even though the systemic exposure  
446 of etoposide increased with higher doses, the absolute bioavailability decreased, and this does not  
447 seem to involve P-gp saturation by etoposide as the absolute bioavailability should then increase.  
448 However, zosuquidar enhanced the oral absorption and bioavailability of etoposide. Yet, in the  
449 presence of 20 mg kg<sup>-1</sup> zosuquidar, only 34.5% of the etoposide dose reached the systemic circulation,  
450 thus zosuquidar itself most likely did not cause full inhibition of intestinal P-gp.

451 Surprisingly, the absolute oral bioavailability of etoposide was low compared to our previous study  
452 (Al-Ali et al., 2018a). Al-Ali and coworkers reported that the oral bioavailability of etoposide was 27

453  $\pm 5\%$  (Al-Ali et al., 2018a). Zhao and co-workers reported an oral bioavailability of etoposide of 25  
454  $\pm 7\%$  in WT male Sprague-Dawley rats dosed with  $12 \text{ mg kg}^{-1}$  etoposide suspended ( $1.5 \text{ mg mL}^{-1}$ ) in  
455 0.5% sodium carboxymethyl cellulose (Zhao et al., 2013). Li and co-workers reported an oral  
456 bioavailability of  $7.5 \pm 1.8\%$  in WT male Sprague-Dawley rats after oral administration of a  $6 \text{ mg kg}^{-1}$   
457 etoposide solution prepared as an injectable formulation (specific composition not specified) (Li et  
458 al., 2009). In the present study, it was not clear why the oral bioavailability of etoposide was low in  
459 the control group, however, besides the known variabilities observed between animal studies (Festing  
460 and Altman, 2002), one could speculate that the 57% of ethanol could be the cause, since etoposide  
461 could precipitate in the intestinal lumen. In the previous study by Al-Ali et al, the oral bioavailability  
462 of etoposide (27%) was obtained after administration of  $20 \text{ mg kg}^{-1}$  in 40% ethanol ( $2 \text{ mg mL}^{-1}$ )  
463 administered with a dosing volume of  $10 \text{ mL kg}^{-1}$  (Al-Ali et al., 2018a). In the present study an  
464 etoposide solution ( $2.86 \text{ mg mL}^{-1}$ ) in 57% (v/v) ethanol at a dose of  $20 \text{ mg kg}^{-1}$  etoposide and a dosing  
465 volume of  $7 \text{ mL kg}^{-1}$  was used, thus approximately similar absolute amounts of ethanol were  
466 administered in the two studies, but the concentration of etoposide and the amount of water differ,  
467 with concentration of etoposide in the previous study being lower and water amount higher. This  
468 could affect the susceptibility of etoposide precipitation in the solution used here to be higher. Ethanol  
469 is rapidly absorbed in rats with a  $t_{\text{max}}$  less than 60 min and 50% disappearance of ethanol from the  
470 stomach after 60 min (Siegers et al., 1972). If etoposide has indeed precipitated, this could explain  
471 the low bioavailability. Population analysis indicated that formulation G decreased both the oral  
472 bioavailability and the absorption rate while a clear increase in oral bioavailability was quantified in  
473 the presence of zosuquidar administration. Introduction of these covariate effects on the respective  
474 parameters of the population pharmacokinetic model significantly decreased the objective function  
475 values (OFVs) and improved the model fit upon inspection of diagnostic plots (see Table 2). It has  
476 been described earlier that 25% (v/v) polysorbate 20 decreased etoposide oral absorption, when

477 similar doses of 20 mg kg<sup>-1</sup> of etoposide were co-administered in rats (Al-Ali et al., 2018a). In the  
478 study reported by Al-Ali et al., dialysis experiments showed that polysorbate 20 at 25% (v/v)  
479 abolished etoposide release across polycarbonate membranes (Al-Ali et al., 2018a). In contrast, the  
480 present study showed that etoposide release from the matrix across polycarbonate membrane was not  
481 affected by the presence of different components in the formulation such as pluronic® F-127. *In vivo*,  
482 this points to an effect of the high ethanol concentration rather than incomplete release from colloidal  
483 structures present in the intestinal lumen.

484

## 485 5. Conclusion

486 In conclusion, an oral formulation of high-concentration of etoposide, which maintained etoposide in  
487 a solubilized form under short-term storage conditions and when diluted with simulated intestinal  
488 fluid was developed. Administration of increasing etoposide doses using this formulation-matrix  
489 enhanced the plasma exposure of etoposide dose-proportionally. However, the applied doses were  
490 not able to saturate P-gp, and the absolute absorption fraction decreased as a function of increasing  
491 dose. To saturate intestinal P-gp by etoposide in rats, oral formulations maintaining high  
492 concentrations of etoposide in solution are needed, yet the present study is a starting point for  
493 developing formulation approaches using other drug candidates aimed at increasing the oral  
494 absorption of P-gp substrates and illustrates the difficulties in developing formulations aimed at  
495 saturating intestinal P-gp transport.

496

497

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514

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672

## 673 **Figures Legends:**

674 **Figure 1. Transepithelial P-gp dependent flux of etoposide across Caco-2 cell monolayers.** The  
 675 steady-state P-gp dependent flux ( $\text{pmol cm}^{-2} \text{ min}^{-1}$ ) of etoposide across Caco-2 cells grown on  
 676 permeable support for 24-28 days was studied as a function of etoposide concentration ( $C_d$ ,  $\mu\text{M}$ ) in  
 677 the donor solution. The A-B transport (**A**) and B-A (**B**) transport was measured in 3 cell passages. In

678 B, the line is obtained by fitting experimental points to Eq. 1 described in Materials and Methods.  
679 Values are given as mean  $\pm$  SEM.

680

681 **Figure 2. Pictures of etoposide formulations after 15 hours of storage at room temperature.**

682 Formulation B contained 12 mg mL<sup>-1</sup> etoposide, 10% (w/v) of pluronic® F-127 in 44% (v/v) ethanol.

683 Formulation G contained 16 mg mL<sup>-1</sup> etoposide, 10% (w/v) of pluronic® F-127, and 10% (w/v) of  
684 PVP/VA in 57% (v/v) ethanol.

685

686 **Figure 3. Absorbance measurements at 400 nm of etoposide formulations added to fasted state**  
687 **simulated intestinal fluid (FaSSIF) in a ratio of (Formulation:FaSSIF, 1:2) as a function of time.**

688 Etoposide formulations were composed as shown in Table 1. The measurements were performed at  
689 room temperature. For formulation G, n=4 and as mean  $\pm$  SEM, for other formulations n=1.

690

691 **Figure 4. Time dependent release of etoposide and glycine from different formulations across**

692 **0.03  $\mu$ m polycarbonate membrane.** Donor solutions of <sup>3</sup>H-etoposide and <sup>14</sup>C-glycine were prepared

693 in Hanks Balanced Salt Solution (HBSS) buffer supplemented with 10 mM HEPES and adjusted to  
694 pH 7.40  $\pm$  0.05 (Control), or in different formulation-matrix containing 10% (w/v) PVP/VA, 57%

695 (v/v) ethanol, and pluronic® F-127 (PF127) at 1.5, 3, or 10% (w/v), referred as Matrix-PF127 1.5%,

696 Matrix-PF127 3%, or Matrix-PF127 10%, respectively, or mixed with formulation G which contained

697 15-16 mg mL<sup>-1</sup> etoposide, 10% (w/v) of PF127 and PVP/VA, and 57% (v/v) ethanol. Receiver-release

698 media were HBSS buffer supplemented with 10 mM HEPES and adjusted to pH 6.50  $\pm$  0.05 for all

699 conditions, except in formulation G condition where the receiver-release media was the matrix of



700 formulation G (i.e. 10% (w/v) of PF127 and PVP/VA, and 57% ethanol, and q.s. water but without  
701 etoposide). Experiments were performed at 37 °C and shaking mode 220 rpm. Data are expressed in  
702 mean  $\pm$  SEM from 3-4 independent membranes and as one membrane per experiment.

703

704

705 **Figure 5. Plasma concentration time profile of etoposide after intravenous administration of 5**  
706 **mg kg<sup>-1</sup> etoposide to wild-type male Sprague-Dawley rats.** Each data point is shown as mean  $\pm$   
707 SEM from 3-6 rats.

708

709 **Figure 6. Plasma concentration time profiles of etoposide after oral administration of different**  
710 **doses of etoposide in wild-type male Sprague-Dawley rats.** A) Etoposide was administered orally  
711 at a dose of 20 mg kg<sup>-1</sup> in 57% (v/v) ethanol solution (Control), or at increasing doses of etoposide  
712 dissolved in matrix at 20, 50, and 100 mg kg<sup>-1</sup> etoposide. The matrix was consisted of 10 % (w/v)  
713 pluronic<sup>®</sup> F-127 and PVP/VA, and 57% (v/v) ethanol, and q.s. water. At the highest oral dose, the  
714 formulation is referred as formulation G. B) Etoposide was administered orally as control after the  
715 rats were received an oral dose 20 mg kg<sup>-1</sup> of zosuquidar. Each data point is shown as mean  $\pm$  SEM  
716 from 5-6 rats. The solid lines are only connecting the data points.

717

718 **Supplementary Figure S1. AUC after oral administration as a function of dose.**

719 Each data point is shown as mean  $\pm$  SEM from 5-6 rats.

720



Table 1: Composition of oral formulations of etoposide. (nd : not determined, ✓: Yes, ÷ : no).

<sup>a</sup>: (Li et al., 2012; Xu and Dai, 2013)

	Formulations							Function
	A	B	C	D	E	F	G	
<b>Composition</b>								
<b>Etoposide (mg mL<sup>-1</sup>)</b>	9	12	12	15	15	15	16	API, P-gp substrate
<b>Pluronic® F-127 (% w/v)</b>	4.7	10	6	6	2	6	10	Surfactant, precipitation inhibitor <sup>a</sup>
<b>PVP/VA (% w/v)</b>	5	-	15	-	5	10	10	Precipitation inhibitor
<b>Ethanol (% v/v)</b>	44	44	57	57	57	57	57	Co-solvent/solvent
<b>Ultra-purified water</b>	qs	qs	qs	qs	qs	qs	Qs	Solvent
<b>Stable under storage for 15 h</b>	✓	÷	✓	✓	✓	✓	✓	
<b>Stable for 2 h after dilution with FaSSIF</b>	nd	nd	÷	÷	÷	÷	✓	

**Table 2: Population pharmacokinetic parameter estimates of the base structural model and the final covariate model.** Precision of the parameter estimates is reported as relative standard error (RSE%). F = oral bioavailability,  $k_a$  = absorption rate, V1 = volume of the central compartment, V2 = volume of the peripheral compartment, CL = elimination clearance, Q2 = intercompartmental clearance,  $\beta_{F,FORM}$  = categorical covariate effect of formulation G presence on F,  $\beta_{F,ZOSU}$  = categorical covariate effect of zosuquidar presence on F,  $\beta_{k_a,FORM}$  = categorical covariate effect of formulation G presence on  $k_a$ , a = additive error model term, b = proportional error model term, OFV = objective function value, -2LL = minus 2 times log likelihood, AIC = Akaike information criterion.

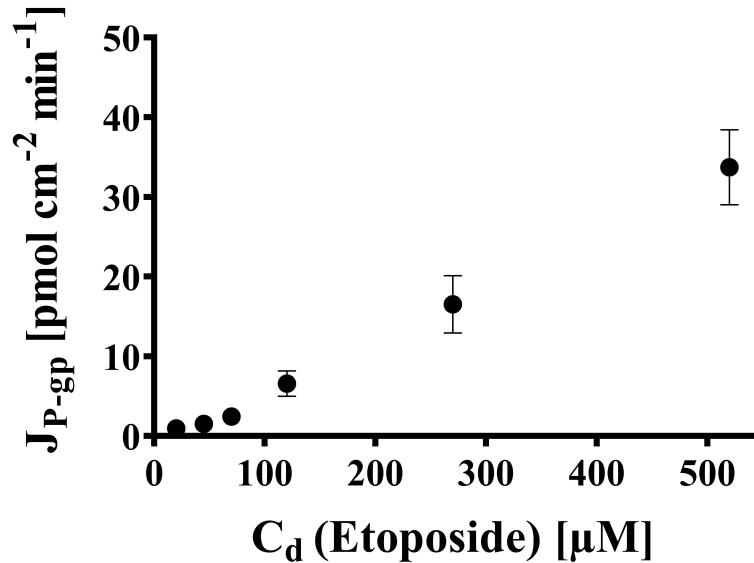
	Base structural model	Final covariate model
<b>Fixed effects [RSE%]</b>		
<b>F (%)</b>	6.76 [24.2]	3.92 [16.7]
<b><math>k_a</math> (h<sup>-1</sup>)</b>	2.69 [13.1]	4.54 [20.2]
<b>V1 (L kg<sup>-1</sup>)</b>	3.63 [18]	2.91 [18.7]
<b>V2 (L kg<sup>-1</sup>)</b>	3.18 [12.2]	3.78 [17.9]
<b>CL (L h<sup>-1</sup> kg<sup>-1</sup>)</b>	1.61 [4.62]	1.55 [10.1]
<b>Q2 (L h<sup>-1</sup> kg<sup>-1</sup>)</b>	6.56 [13.6]	6.98 [18.6]
<b><math>\beta_{F,FORM}</math></b>	-	-0.51 [37.9]
<b><math>\beta_{F,ZOSU}</math></b>	-	2.17 [7.04]
<b><math>\beta_{k_a,FORM}</math></b>	-	-0.713 [34.8]
<b>Random effects (SD) [RSE%]</b>		
<b>F</b>	1.1 [13.4]	0.158 [28.1]
<b><math>k_a</math></b>	0.552 [20.6]	0.502 [23.2]
<b>V1</b>	0.924 [13]	0.678 [15]
<b>CL</b>	0.122 [35.6]	0.118 [38.8]
<b>Error model [RSE%]</b>		
<b>a</b>	0.01 [14.1]	0.0115 [14.5]
<b>b</b>	0.132 [9.34]	0.135 [10.9]
<b>OFV</b>		
<b>-2LL</b>	-665.17	-734.78
<b>AIC</b>	-641.17	-704.78

**Table 3: Estimated pharmacokinetic parameters after intravenous and oral administration of different doses of etoposide in wild-type male Sprague-Dawley rats.** Matrix solution consisted of 10 % (w/v) pluronic<sup>®</sup> F-127, 10% (w/v) PVP/VA, 57% (v/v) ethanol, and q.s. water. Individual Bayesian (post hoc) estimates were used to calculate mean AUC<sub>0-∞</sub>. Data are expressed as mean ± SEM except for t<sub>max</sub> that is expressed as the median [Q1; Q3] (25% and 75% percentile). No random effects were estimated for Q and V<sub>2</sub>. Fixed effects were estimated as Q = 6.98 mL h<sup>-1</sup> kg<sup>-1</sup>, V<sub>2</sub> = 3778 mL kg<sup>-1</sup>. Statistical significance was tested by one-way ANOVA followed by Tukey's multiple comparisons test. When all the groups, except the group of rats pre-dosed with zosuquidar and the group of rats receiving i.v. administration, were included in the comparison, (\*) referred to P < 0.05 compared to control. When all the groups, except the group of rats receiving i.v. administration, were included in the comparison, (†) referred to P < 0.05 compared to control.

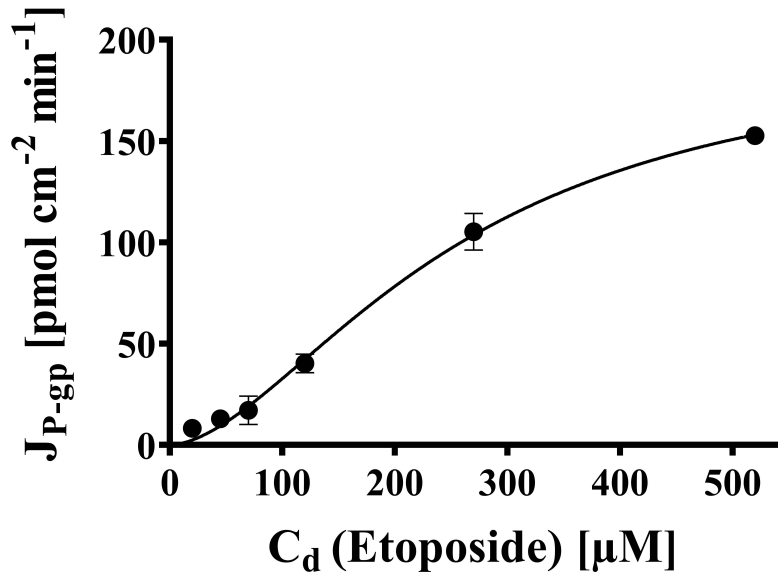
Study cohorts						
<b>Etoposide dose</b>	20 mg kg <sup>-1</sup>	20 mg kg <sup>-1</sup>	50 mg kg <sup>-1</sup>	100 mg kg <sup>-1</sup>	20 mg kg <sup>-1</sup>	5 mg kg <sup>-1</sup>
<b>Condition</b>	Control				Zosuquidar	
<b>Etoposide solution</b>	57% EtOH	Matrix	Matrix	Matrix	57% EtOH	57% EtOH
<b>Cohort Size</b>	6	5	6	6	6	6
<b>Administration route</b>	p.o.	p.o.	p.o.	p.o.	p.o.	i.v.
<b>F (%)</b>	4.0 ± 0.13	2.4 ± 0.02*	2.4 ± 0.07*	2.2 ± 0.09*	34.5 ± 0.18 <sup>†</sup>	100 ± 0
<b>k<sub>a</sub> (h<sup>-1</sup>)</b>	6.40 ± 0.94	2.29 ± 0.41* <sup>†</sup>	2.36 ± 0.35* <sup>†</sup>	2.32 ± 0.32* <sup>†</sup>	3.43 ± 0.41 <sup>†</sup>	-

<b>CL (mL h<sup>-1</sup> kg<sup>-1</sup>)</b>	1547 ± 28	1533 ± 6.9	1503 ± 24	1587 ± 39	1550 ± 43	1636 ± 85
<b>V<sub>1</sub> (mL kg<sup>-1</sup>)</b>	3830 ± 574	4263 ± 193	4143 ± 315	4953 ± 640	2996 ± 436	809 ± 50
<b>AUC<sub>0-∞</sub> (ng h mL<sup>-1</sup>)</b>	518.8 ± 25	307.2 ± 3.5*	816.5 ± 38*	1525 ± 86*	4511 ± 384 <sup>†</sup>	3096 ± 155
<b>AUC<sub>0-∞</sub>/D (h mL<sup>-1</sup> kg)</b>	25.9 ± 1.3	15.4 ± 0.2	16.3 ± 0.8	14.2 ± 0.9	225.5 ± 19.2	619.2 ± 31.0
<b>t<sub>1/2</sub> (h)</b>	1.73 ± 0.27	1.93 ± 0.08	1.91 ± 0.14	2.18 ± 0.31	1.36 ± 0.23	0.35 ± 0.02
<b>t<sub>max</sub> (h)</b>	0.375 [0.25-0.5]	0.625 [0.44-0.75]	0.75 [0.25-1.06]	0.75 [0.5-1.25]	0.5 [0.5-0.81]	-
<b>C<sub>max</sub> (ng mL<sup>-1</sup>)</b>	191 ± 19	63 ± 4*	169 ± 17	266 ± 38*	1542 ± 190 <sup>†</sup>	-

**A) A-B**

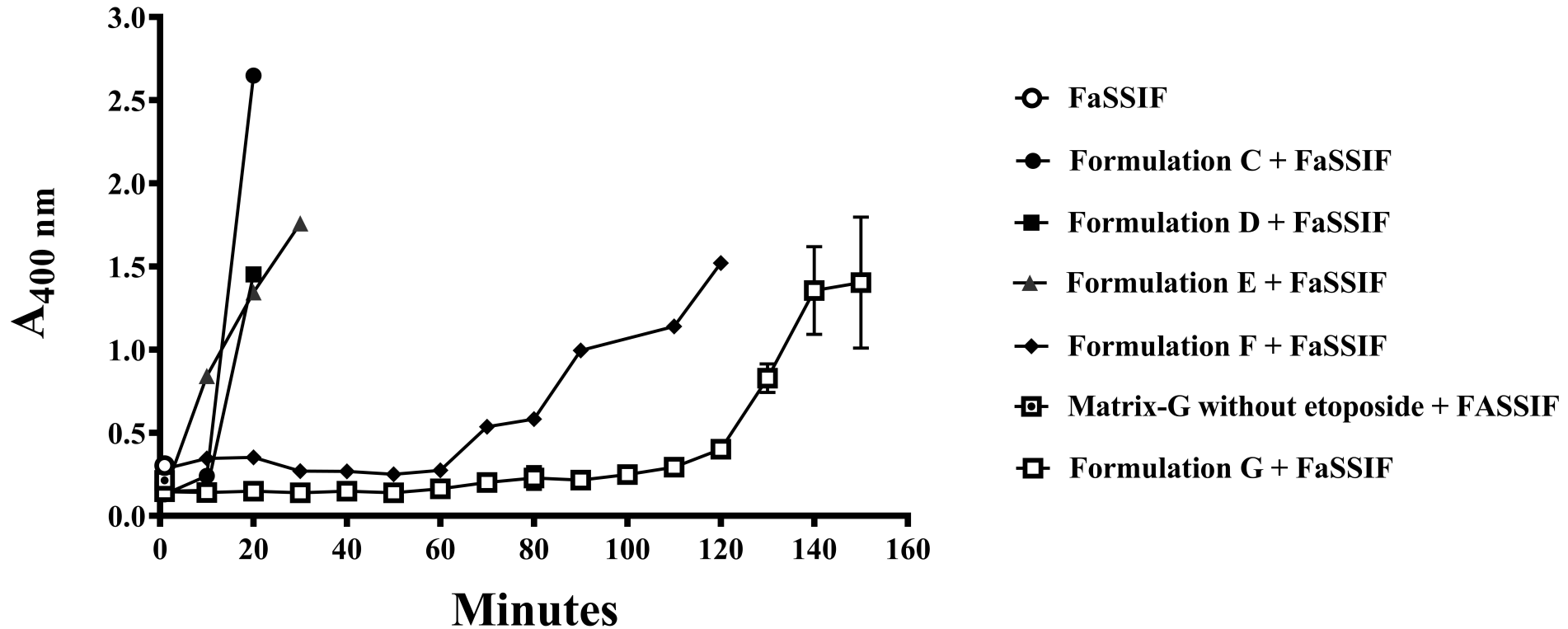


**B) B-A**

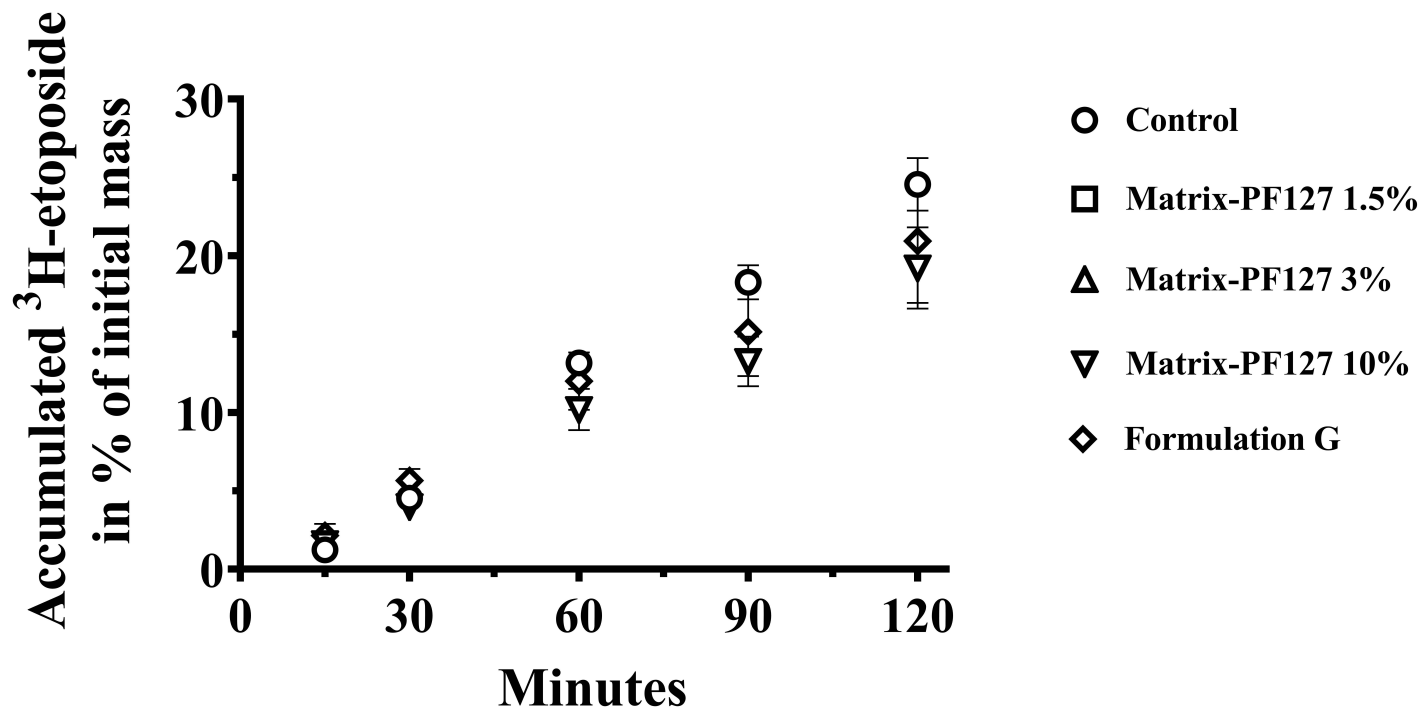




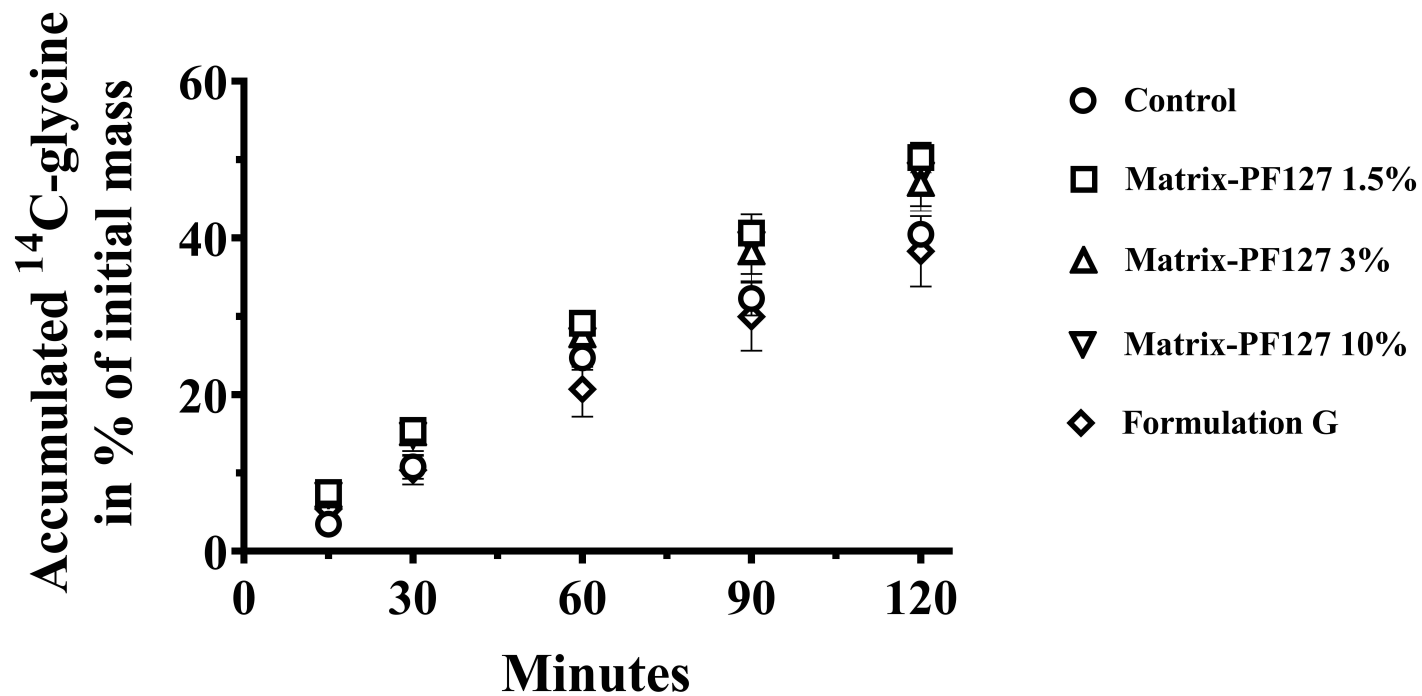


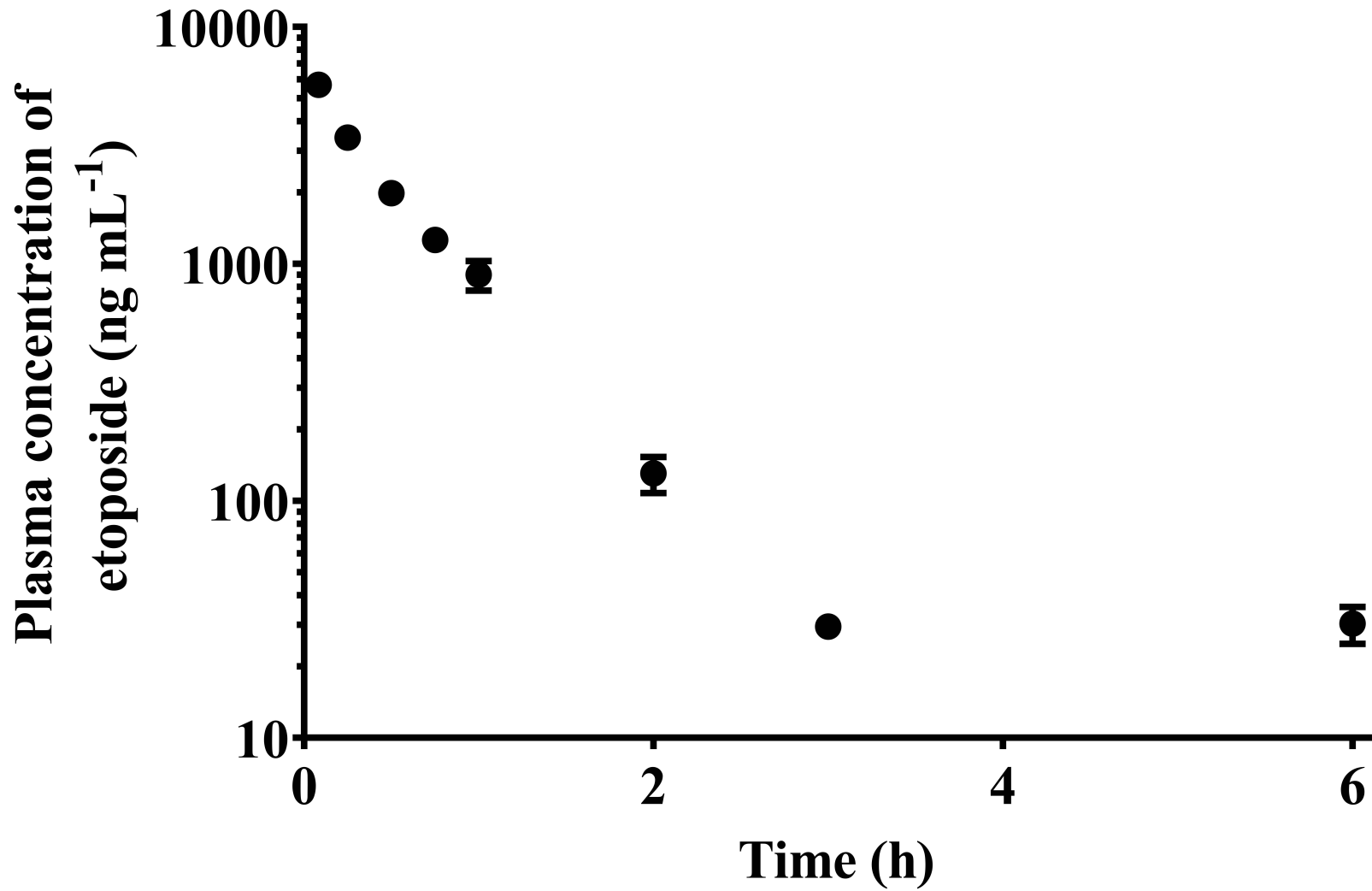


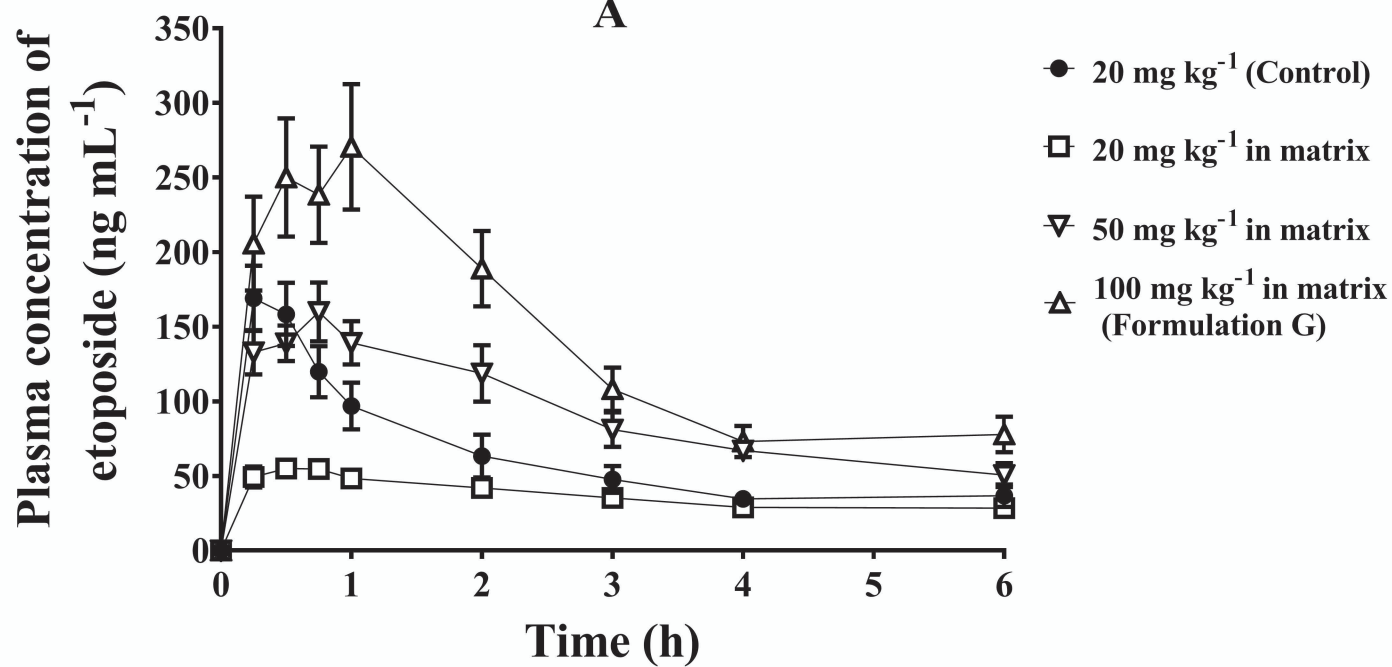
(A)



(B)





**A****B**