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1	Exploring the impact of surfactant type and
2	digestion: Highly digestible surfactants improve
3	oral bioavailability of nilotinib
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22 TABLE OF CONTENT



24

25 ABSTRACT

26 The scientific rational for selection of surfactant type during oral formulation development 27 requires an in-depth understanding of the interplay between surfactant characteristics and 28 biopharmaceutical factors. Currently, however, there is a lack of comprehensive knowledge of 29 how surfactant properties, such as hydrophilic-lipophilic balance (HLB), digestibility and fatty 30 acid (FA) chain length, translate into *in vivo* performance. In the present study, the relationship 31 between surfactant properties, in vitro characteristics and in vivo bioavailability was 32 systematically evaluated. An in vitro lipolysis model was used to study the digestibility of a 33 variety of non-ionic surfactants. Eight surfactants and one surfactant mixture were selected for 34 further analysis using the model poorly water-soluble drug nilotinib. In vitro lipolysis of all 35 nilotinib formulations was performed followed by an *in vivo* pharmacokinetic evaluation in 36 rats. The *in vitro* lipolysis studies showed that medium chain FA based surfactants were more 37 readily digested compared to long chain surfactants. The in vivo study demonstrated that a Tween 20 formulation significantly enhanced the absolute bioavailability of nilotinib up to 5.2-38 39 fold relative to an aqueous suspension. In general, surfactants that were highly digestible in

Page 2

40	vitro tended to display higher bioavailability of nilotinib in vivo. The bioavailability may
41	additionally be related to the FA chain length of digestible surfactants with an improved
42	exposure in the case of medium chain FA based surfactants. There was no apparent relationship
43	between the HLB value of surfactants and the in vivo bioavailability of nilotinib. The impact
44	of this study's findings suggests that when designing surfactant-based formulations to enhance
45	oral bioavailability of the poorly water-soluble drug nilotinib, highly digestible, medium chain-
46	based surfactants are preferred. Additionally, for low permeability drugs such as nilotinib,
47	which is subject to efflux by intestinal P-glycoprotein, the biopharmaceutical effects of
48	surfactants merit further consideration.
49	
50	KEYWORDS
51	Surfactants; Digestibility; Nilotinib; Poorly water-soluble drugs; Suspension; Lipolysis
52	
53	ABBREVIATIONS
54	AUC, area under the curve; CMC, critical micellar concentration; CYP, cytochrome P450; FA,
55	fatty acid; FFA, free fatty acids; HLB, hydrophilic-lipophilic balance; P-gp, P-glycoprotein
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61 INTRODUCTION

62 Many emerging drug candidates show poor solubility and/or permeability resulting in a low and variable oral bioavailability when administered in conventional dosage forms.¹ Therefore, 63 64 there is a need to develop bio-enabling formulation technologies that enhance biopharmaceutical properties and improve oral absorption of these emerging drug candidates. 65 2 The various bio-enabling approaches have been extensively reviewed including solid 66 dispersions, ^{3, 4} lipid-based formulations ⁵ and nano-sized drug crystals/particles. ⁶ 67 Interestingly, one of the most common excipients included across most classes of bio-enabling 68 69 approaches are surfactants, which, from a mechanistic perspective, can impart a variety of biopharmaceutical advantages including promoting supersaturation, ^{7, 8} enhancing 70 solubilisation, ⁹ stabilisation of colloidal/nano-crystals, ¹⁰ increased dissolution rate ¹¹ and 71 increasing permeability. 12-16 72

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74 Surfactants can be classified according to the polar head group into ionic (cationic, anionic or zwitterionic) or non-ionic surfactants. ¹⁷ Non-ionic surfactants are considered favourable due 75 76 to a low toxicity and a more readily maintained solubilising power under biorelevant conditions. ¹⁸ The most commonly used non-ionic surfactants are based on ethylene oxide / 77 polyoxyethylene and referred to as ethoxylated surfactants e.g. sorbitan ester ethoxylates or 78 fatty amine ethoxylates. ¹⁹ Other important non-ionic surfactant classes include polyol-based 79 80 surfactants (e.g. glycoside, glycol or glycerol esters), amine oxides and sulfinyl surfactants.¹⁹ 81 Ethoxylated and polyol surfactants can be further sub-classified into esters (e.g. glycol, 82 glycerol, sorbitan, fatty acid ethoxylates) and ethers (e.g. poloxamers, ethoxylated fatty 83 alcohol, alkyl phenol ethoxylates). For pharmaceutical applications ethoxylated and polyol 84 esters and ethers such as Tweens (ethoxylated sorbitan esters) or Poloxamers (ethylene oxidepropylene oxide copolymers) are widely utilised. As an excipient class, surfactants can exhibit 85 Page 4

a diverse set of properties and characteristics, and hence a variety of physiochemical 86 87 approaches have been applied for characterising surfactants. These include properties such as hydrophilic-lipophilic balance (HLB) value, molecular weight, chain length, molecular 88 volume, critical micellar concentration (CMC), solubility parameters. ²⁰ While many of these 89 90 properties have been explored for a specific formulation approach, to date the selection of 91 surfactants remains mostly empirically driven. Additionally, while many surfactants are 92 derived from digestible fats and oils, the impact of digestion on surfactants is commonly not 93 considered in formulation performance. However, digestibility of surfactants can impact many 94 of the aforementioned physico-chemical properties of the excipient, as the chemical structure 95 can change along the transit though the GIT. Furthermore, this illustrates the difficulty in 96 developing a reliable surfactant classification system as properties of the surfactants may be influenced by *in vivo* conditions. ¹⁸ The present study aimed to address knowledge gaps in the 97 98 literature on the relationships between surfactant properties and the biopharmaceutical 99 performance in vivo. This study, therefore, provides a basis for establishing a performance-100 based classification of surfactants in oral drug delivery.

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102 In addition to the solubility enhancing effects, surfactants can interact with lipid bilayer of cell membranes thereby increasing the permeability. ¹² Furthermore, surfactants may influence pre-103 systemic clearance of drugs by modulating transporters and metabolising enzymes. ¹³ For 104 105 example, Tween 80 and Cremophor EL increased the uptake of digoxin, a P-glycoprotein (P-106 gp) substrate, to the same extent as cyclosporin (a commonly used P-gp inhibitor), using the rat everted gut sac method. ¹⁴ This indicated that Cremophor EL and Polysorbate 80 can 107 108 modulate P-gp to improve the bioavailability. Additionally, Pluronic F68, Labrasol, Brij 30 109 and Tween 20 have also shown to be inhibitors of P-gp in vitro using the rat everted gut sac method or various cell lines. ^{15, 16} In addition to modulation of P-gp efflux activity, a number 110 Page 5 of surfactants such as Tween 20, Cremophor EL, Pluronic F68 and Myrj S40 have demonstrated inhibitory effects on cytochrome P450 3A4 (CYP 3A4), ²¹ a key metabolic enzyme present in intestinal tissue. In the latter study, it was shown that among the tested surfactants Tween 20 showed the highest inhibition resulting in a significantly increased area under the curve (AUC) and a 40% decrease in AUC of the main metabolite of midazolam in rats. ²¹

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118 When considering the choice of digestible versus non-digestible surfactants, a key perceived 119 advantage of non-digestible surfactants is in being 'digestion-independent' systems, and 120 therefore *in vitro* characteristics can be readily employed to predict the likely performance *in* 121 vivo. Additionally, digestible systems carry the risk that the surfactant may not serve the initial 122 purpose in a post-digestive state. For example, digestion of surfactants may lead to a reduced 123 solubilisation capacity of the colloidal aqueous dispersion and potentially lead to drug 124 precipitation. However, studies have also suggested that digestion may in fact promote transient supersaturation in the intestinal media and therefore may promote absorption.²² In 125 addition, the released FA have shown to increase solubility of poorly soluble drugs as 126 127 evidenced by *in vitro* measurements of solubility in assembled pre- and post-digestion media with lipid excipients. ^{23, 24} Moreover, free fatty acids (FFA) released post digestion may 128 129 modulate the intestinal permeability. In several studies it was shown that the treatment of cells 130 with medium chain FFA showed an increased paracellular transport via tight junction opening. 131 ²⁵⁻³⁰ In the case of unsaturated long chain FFA, *in vitro* cell experiments showed an increased membrane fluidity ³¹⁻³³ as well as opened tight junctions. ^{26, 34} These biopharmaceutical effects 132 133 can further enhance drug absorption from surfactant formulations.

135 There is currently a lack of comprehensive knowledge of how surfactant properties translate 136 into in vivo bioavailability of poorly water-soluble drugs. The overarching goal of this study was to systematically explore the relationship between surfactant properties and in vivo 137 138 performance, which will support a more science- and risk-based approach to surfactant 139 selection in oral formulations. Nilotinib was chosen as a model poorly water-soluble drug for 140 the study. Nilotinib is practically insoluble in buffer solutions of pH 4.5 and higher (pKa 2.1 and 5.4), ³⁵ is highly lipophilic (log P 4.95) as well as hydrophobic ²⁴ and exhibits a moderate 141 permeability across a confluent Caco-2 cell monolayer. ^{35, 36} Therefore, nilotinib was 142 143 categorised as a class IV compound in the biopharmaceutical classification system (BCS). Nilotinib is predominantly metabolised by CYP 3A4³⁵ and is a P-gp substrate. ³⁵⁻³⁸ The pre-144 145 systemic clearance is high with an AUC increase of 29% after co-administration of nilotinib with grapefruit juice (intestinal CYP 3A4 inhibitor).³⁹ The current commercial formulation, 146 Tasigna®, is a capsule formulation containing the surfactant Pluronic F68 (poloxamer 188).⁴⁰ 147 148 The concentration and the scientific rational of the addition of Poloxamer 188 is unknown, 149 however it has been reported that the use of surfactants did not increase the dissolution of nilotinib capsules at pH 4.5 and above. ³⁶ The marketed formulation of nilotinib showed an 150 absorption of $\geq 30\%$ following a radiolabelled single 400 mg oral dose in humans.⁴¹ 151 Additionally, preclinical studies in rats yielded an absolute bioavailability of 34% using a 152 solution with Cremophor, dimethyl acetamide and 5% dextrose (20/10/70 (v/v/v)). ³⁵ Using 153 154 this as a model poorly water-soluble drug, the present study systematically assessed the impact 155 of surfactant properties on oral bioavailability. Surfactants were classified according to FA 156 chain length, HLB value and digestibility and their in vitro and in vivo performance in rats was 157 investigated.

159 MATERIALS AND METHODS

160 *Chemicals and materials*

Nilotinib and sorafenib were purchased from Kemprotec Ltd. (UK). Brij O2 (Polyoxyethylene 161 162 (2) olevl ether), Brij L23 (Polvoxyethylene (23) lauryl ether), Myrj S40 (Polyoxyethylene (40) 163 stearate), Span 85 (Sorbitan trioleate), Span 80 (Sorbitan monooleate), Tween 80 164 (Polyoxyethylene sorbitan monooleate) and Tween 20 (Polyoxyethylene sorbitan monolaurate) 165 were kindly donated by Croda international Plc (UK). Lipoid E PC S (Phosphatidylcholine) 166 was gifted by Lipoid GmbH (Germany). Taurodeoxycholic acid sodium salt (NaTDC), 167 pancreatic lipase (8 x USP), Cremophor RH40 (Polyoxyl 40 hydrogenated castor oil) and 168 Tween 85 (Polyoxyethylene sorbitan trioleate) were ordered from Sigma-Aldrich (Ireland). A 169 sample of Labrasol (Caprylocaproyl polyoxyl-8 glycerides), Labrafil M1944 CS (Oleoyl 170 polyoxyl-6 glycerides), Labrafil M2125 CS (Linoleoyl polyoxyl-6 glycerides), Plurol Oleique 171 CC 497 (Polyglyceryl-3 dioleate) and Gelucire 44/14 (Lauroyl polyoxyl-32 glycerides) was 172 kindly donated by Gattefossé (France). A sample of Cremophor EL (Polyoxyl 35 castor oil) was kindly donated by BASF (Germany). All other chemicals and solvents were of analytical 173 174 or high-performance liquid chromatography (HPLC) grade and were purchased from Sigma-175 Aldrich (Ireland) and used as received.

176

177 Solubility studies

Equilibrium solubility at 37 °C was determined in Tween 20, Tween 85, Labrasol, Labrafil
M1944CS, Span 80, Cremophor RH40, Brij O2, Brij L23, and in a Tween 85/Cremophor RH40
mixture (67:33 w/w) in order to represent the formulations that were used during *in vitro*lipolysis and the *in vivo* study. Solid excipients were melted at 50 °C and cooled to 37 °C, while
liquid surfactants were heated to 37 °C before addition of excess nilotinib. The suspensions
were stirred at 250 rpm and 37 °C. Samples were taken after 24 h, 48 h, 72 h and centrifuged Page 8

at 21,380g and 37 °C for 15 min (Mikro 200 R, Hettich GmbH, Germany). The supernatant was transferred to a new tube and centrifuged again under identical conditions. To solubilise the surfactants, the supernatant was diluted in acetonitrile, ethyl acetate (1:4, v/v). Followed by further 1:10 (v/v) dilution with acetonitrile, ethyl acetate (4:1). The obtained samples were diluted appropriately with mobile phase before analysis by reverse phase HPLC. Equilibrium was assumed once two time-points did not differ more than 10%. All samples were run in triplicates.

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The samples were analysed as described previously.²⁴ In brief, an Agilent 1200 series HPLC 192 193 system comprised a binary pump, degasser, autosampler and variable wavelength detector. 194 Data analysis was done with EZChrom Elite version 3.2. Nilotinib was separated with a Zorbax 195 Eclipse Plus-C18 column (5 µm, 4.6 mm x 150 mm) including a Zorbax Eclipse Plus-C18 196 guard column (5 µm, 4.6 mm x 12.5 mm) at 25 °C. The mobile phase consisted of acetonitrile, 197 methanol, water and triethylamine (35:30:34:1 v/v) and was used at a flow rate of 0.9 mL/min. 198 20 µL samples were injected and the detection wavelength was 267 nm. The limit of detection 199 (LOD) and limit of quantification (LOQ) of this method was 4 ng/mL and 12 ng/mL, 200 respectively. Values were determined using the standard error of y-intercept according to the ICH Q2 guideline 42 and linearity was confirmed between 12 ng/mL and 12 µg/mL drug 201 202 concentration.

203

In vitro lipolysis: Digestibility and drug solubilisation during formulation dispersion and
 digestion

In vitro lipolysis was studied using a pH-stat apparatus (Metrohm AG, Herisau, Switzerland)
comprising a Titrando 907 stirrer, 804 Ti-stand, a pH electrode (Metrohm, Herisau,
Switzerland) and two 800 Dosino dosing units coupled to a 20 mL autoburette. The system was
Page 9

209 operated by the Tiamo 2.2 software. The in vitro protocol was amended from Williams et al. ^{43, 44} In brief, the buffer contained 2 mM TRIS maleate, 150 mM NaCl, 1.4 mM CaCl₂·2H₂O, 210 211 adjusted to pH 6.5. For the digestion experiments the buffer was supplemented with 3 mM 212 NaTDC and 0.75 mM PC and stirred for 12 hours before further usage. The pancreatin extract 213 was prepared freshly by adding 5 mL of 5 °C buffer to 1 g of porcine pancreatic enzymes (8x 214 USP), which was vortexed thoroughly. The mixture was centrifuged for 15 min at 5 °C, 2800g (Hettich Rotina 380R) and 4 mL of supernatant was recovered and stored at 2 - 8 °C before 215 216 further usage. The pancreatic extract had a pancreatic lipase activity of $\sim 10\,000$ TBU/mL (to 217 provide approximately 1000 TBU per mL of digest), where 1 TBU represents the amount of enzyme that liberates 1 µmol of FA from tributyrin per min. ⁴⁵ All experiments were conducted 218 219 with a stirring speed of 450 rpm.

220

For the digestibility study, 1.0 g of blank excipient was dispersed into 36 mL of digestion 221 buffer. The pH was adjusted to 6.5 using 0.2 M, 0.6 M or 1 M NaOH depending on the pH 222 223 change upon excipient addition. Digestion was initiated by the addition of 4 mL pancreatic 224 enzyme and the pH of 6.5 was maintained using 0.2 M and 0.6 M NaOH for long and medium 225 chain excipients, respectively. After 60 min of digestion the enzymes were inhibited using 1 M 4- bromophenylboronic acid in methanol (5 µL per mL media) and the pH was increased to 226 227 9.0. An additional blank titration using solely the digestion buffer was performed and the 228 released mmol of FFAs from the blank was subtracted from the mmol of FFAs released from 229 the surfactant formulations. The determined amount of FFA was assumed as a surrogate 230 parameter for digestibility. Additionally, the % digested was calculated using the theoretical 231 released FFAs per g of excipient:

232
$$Theoretical FFA [mmol] = \frac{SV [mg]}{56.1056 [\frac{g}{mol}]}$$
(1)

where FFA are the FFAs that can theoretically be released from the excipient in mmol per g of excipient, SV the saponification value in mg KOH per g of excipient from the certificate of analysis and 56.1056 g/mol the molecular weight of KOH. The absolute amount of theoretical possible released mmol FFA can be calculated by multiplying by the amount of excipient used in this study, i.e 1.0 g. The % digested can be calculated as follows:

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$$\% \ digested = \frac{Released \ FFA \ [mmol]]}{Theoretical \ FFA \ [mmol]]} \times 100 \ \%$$
(2)

where the released FFA are the total mmol of FFA released in the digestibility experiment (including the amount detected during back titration to pH 9.0) and theoretical FFA the total mmol of FFA calculated with equation 1.

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For the *in vitro* lipolysis experiment with nilotinib 1.075 g of lipid formulation was dispersed into 39 mL of digestion buffer for 10 min. Three 1 mL samples were taken at 2.5, 5 and 10 min from the middle of the vessel. pH of the media was adjusted and maintained at 6.5 using 0.2 M NaOH and 0.6 M NaOH for medium and long chain excipients, respectively. To the remaining 36 mL (1 g lipid formulation) dispersion 4 mL of pancreatin extract was added to initialize digestion. After 60 min the released non-ionized FFAs were determined by a pH increase of the buffer to pH 9.

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Samples of 1 mL were taken at 5, 10, 15, 30, 45 and 60 min during the digestion experiment from the middle of the vessel. In each sample and after 60 min the enzymes were inhibited by the addition of 1 M 4-bromophenylboronic acid in methanol (5 μ L per mL sample). Additionally, to each 1 mL sample during digestion a 100 μ L sample was taken and added to 900 μ L of acetonitrile and methanol (35:30 v/v) and mixed. This sample was used to quantify the total drug recovery, which allowed adjustments of inhomogeneous samples. All samples
were centrifuged at 37 °C and 21,000g for 30 min (Hettich Micro 200R).

- 258
- 259 Formulations for in vivo and in vitro studies

The solubility of nilotinib in the studied surfactants was low resulting in high dose solubility 260 261 ratios (Table 1) and therefore it was not possible to prepare surfactant solutions at the target dose. It was hence decided to use surfactant suspensions for all experiments, keeping the 262 263 surfactant and dose load constant. The surfactant suspensions were prepared by combining 10 264 mg nilotinib with 1 mL surfactant excipient followed by an over-night stir at 37 °C resulting in varying fractions of pre-dissolved nilotinib (Table 1). The suspensions were stirred constantly 265 266 to prevent sedimentation before usage. Solid excipients were melted prior to nilotinib addition 267 and kept in a liquid form while stirring, addition and *in vivo* administration.

Table 1 Dose:solubility ratio of studied surfactant formulations. The dose was fixed at
10 mg/mL.

Excipient	Dose:solubility ratio			
Span 80	17.86			
Brij O2	14.49			
Labrafil M1944 CS	11.36			
Tween 85	7.87			
Tween 85/ Cremophor RH40 67:33 (w/w)	5.32			
Tween 20	4.83			
Brij L23	4.69			
Cremophor RH40	2.94			
Labrasol	2.43			

Formulations for in vitro and in vivo studies

271

272 In vivo study

273 The protocol used for the in vivo pharmacokinetic study was approved by the institutional 274 animal ethics committee in accordance with Belgian law regulating experiments on animals 275 and in compliance with EC directive 2010/63/EU and the NIH guidelines on animal welfare. 276 Male Sprague-Dawley rats weighing 280-320 g (8-10 weeks of age) on the day of the 277 experiments were purchased from Charles River Laboratories Deutschland (Sulzfeld, 278 Germany) and maintained on standard food and water ad libitum in the laboratory for at least 279 5 days before entering the experiment. Food was removed 16-20 h before dosing and water 280 was available *ad libitum* at all times. Parallel groups of animals were administered with each formulation at a volume of 2 mL/kg by oral gavage with a nilotinib dose of 20 mg/kg. By 281

individual tail vein puncture, 200 μ L blood samples were collected into plasma collection tubes containing dipotassium EDTA. Samples were taken at 0.5, 1, 2, 4, 6, 8, 10 and 24 h following oral dosing. Plasma was harvested immediately by centrifugation for 10 min at 1,000 *g* and stored at -80 °C until analysis. After the experiment the animals were euthanized.

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287 Bioanalysis

288 The plasma concentrations of nilotinib were determined by reversed phase HPLC. The Agilent 289 1260 series HPLC system comprised a binary pump, degasser, temperature controlled 290 autosampler, column oven and diode array detector. The system was controlled, and the data analysed with EZChrom Elite version 3.3.2. The used method was described earlier. ²⁴ In brief, 291 292 a Zorbax Eclipse Plus-C18 column (5 µm, 4.6 mm x 150 mm) with a Zorbax Eclipse Plus-C18 293 guard column (5 µm, 4.6 mm x 12.5 mm) was used. The mobile phase consisted of water, 294 methanol, acetonitrile and triethylamine (34:30:35:1 v/v) and was used at a flow rate of 0.9 295 mL/min. The sample and column temperature were set at 5 °C and 25 °C, respectively, and the 296 detection wavelength was 267 nm. Nilotinib was extracted from the plasma samples by liquid-297 liquid extraction. To 50 μ L of the plasma sample 66 μ L of a methanol acetonitrile mixture 298 (30:35 v/v), containing 1.25 µg/mL sorafenib as internal standard, was added. The mixture was 299 mixed thoroughly and centrifuged at 22 °C, 11,500g for 9 min. 50 µL of the supernatant was 300 injected to the HPLC system for analysis. The LOD and LOQ values of this method in plasma 301 were 11 ng/mL and 37 ng/mL, respectively, as determined using the standard error of yintercept according to the ICH Q2 guideline. ⁴² Linearity was confirmed between 37 ng/mL 302 303 and 4.1 μ g/mL drug concentration.

305 Data Analysis

The pharmacokinetic parameters were calculated using Microsoft Excel. The plasma concentration profiles were analysed by non-compartmental analysis and calculation of AUC by the linear trapezoidal rule. Absolute bioavailability was calculated using previous reported intravenous data. ⁴⁶ The statistical analysis for all *in vivo* parameters was performed using a one-way analysis of variance (one-way ANOVA) after using the Bartlett's test to check for equal variance. Groups were pairwise compared using Tukey's multiple comparison test.

A one-way ANOVA was also calculated for the lipolysis data using the Tukey post-hoc test to
compare the different formulation performances. All statistical analyses were carried out using
GraphPad Prism 5.

315

316 RESULTS

317 Ranking the digestibility of lipid-based surfactants

The digestibility of 15 surfactants and one surfactant blend commonly used in oral drug formulations was assessed using the *in vitro* lipolysis model. A variety of both medium and long chain FA were selected, with HLB values ranging from 2 to 17. The studied surfactants included stearate, oleate or palmitate, which were considered as long chain FA. Surfactants consisting of FAs like caprylic, capric or lauric acid were considered as medium chain excipients. The FFA based digestibility is shown in Figure 1 A and the % digested based digestibility is shown in Figure 1 B.



Figure 1. Surfactant digestibility in the *in vitro* lipolysis test. Tween/Cremophor is a mixture of Tween 85 and Cremophor RH40 at a ratio of 67:33 (w/w). The shaded area represents long chain excipients and non-shaded areas represent medium chain excipients. The numbers above the bars are the corresponding HLB values of the surfactants. Data is presented as mean \pm SD, where n=3. A: Free fatty acids (FFA) released. The total FFA released is divided into the

amount of FFA released during pH stat titrated directly at pH 6.5 (white bars) and the
determined amount of FFA during increase of the pH to pH 9.0 after 60 min of digestion (back
titration, dotted bars). B: % digested based on the theoretical possible amount of FFA release.

336 In general, medium chain-based surfactants displayed the highest quantity of FFA released. 337 Labrasol, a medium chain excipient mainly consisting of PEG-8 mono- and diesters of caprylic 338 and capric acid showed the highest release of FFA (Figure 1 A). Gelucire 44/14, which is 339 composed of mostly PEG-32 mono- and diesters of lauric acid, showed the second highest 340 amount of released FFA. Tween 20, a polyoxyethylene sorbitan monoester of lauric acid, 341 released 0.63 ± 0.04 mmol of FFA (Figure 1 A), which reflected a theoretical digestion of 76% 342 (Figure 1 B). Brij L23, a polyoxyethylene lauryl ether, was included as a negative control, as the ether cannot be digested by lipases (a subclass of esterases) ^{47, 48} and therefore, showed the 343 344 expected absence of FFA release.

345

346 Among the long chain surfactants, Labrafil M1944 CS, which consists of mono-, di- and 347 triglycerides and PEG-6 mono- and diesters of oleic acid, showed the highest released FFA 348 (Figure 1 A). Considering the theoretically possible digestion, only $33.8 \pm 1.0\%$ of Labrafil 349 M1944 CS was digested (Figure 1 B). The highest % digested was shown for Tween 80, a 350 surfactant that like Labrafil M1944 CS also contains oleic acid (Figure 1 B). While the 351 lowest % digested was observed for Span 85 (Figure 1 B), the lowest amount of FFA was 352 released in the case of Cremophor RH40 (Figure 1 A). This finding agreed with previous 353 studies that demonstrated a low release of FFAs as well as theoretical digestibility of Cremophor RH40. ^{49, 50} Brij O2, a polyoxyethylene olevl ether, did not undergo digestion and 354 was included as a negative control. In fact, long chain surfactants were digested to a lesser 355 356 extent compared to medium chain surfactants.

357 Based on the digestibility properties observed, surfactants with a range of digestibility, HLB 358 value and chain length were selected for further in vitro and in vivo investigations. The study 359 design allowed the evaluation of the influence of digestion, HLB value and FA chain length on 360 the formulation performance in vivo (Figure 2). In terms of ranking digestibility, FFA released 361 was chosen as a surrogate parameter for how much the system is changing over time rather 362 than the % digested, as surfactants can show inhibitory effects on lipolysis and the excipient and its lipolytic products can exhibit different activities on the digestive enzymes. ^{51, 52} 363 364 Especially in cases with a high amount of possible released FAs the adjustment to % digested 365 may lack information on the amount of liberated FA, which are crucial for the beneficial 366 solubilising and biopharmaceutical effects. Labrasol, Labrafil M1944 CS, Tween 20, Span 80, 367 Brij L23 and Brij O2 were selected as the digestibility was matching with the chain length and 368 HLB value, respectively. Additionally, the two widely used surfactants Tween 85 and 369 Cremophor RH40, as well as a 2:1 (w/w) mixture of both, were included in the *in vivo* study. 370 The study design is shown in Figure 2 and the surfactant properties in Table 2.





Figure 2. Selected excipients for further *in vitro* and *in vivo* evaluation. Excipients were
selected according to the fatty acid chain length, HLB and total free fatty acids (FFA) released
during the lipolysis experiments as a surrogate parameter for digestibility.

376 *Comparing nilotinib solubility as a function of lipid-based surfactant type*

The solubility of nilotinib in all selected excipients was measured at 37 °C. The results of the solubility studies are presented in Table 2 and Figure S 1 in the supporting information. Nilotinib displayed the highest solubility in Labrasol and the lowest in Span 80. There were no apparent trends in relationship between solubility and either the FA chain length nor HLB value. **Table 2.** Surfactant properties, solubility of nilotinib in surfactants, nilotinib solubilisation in the aqueous phase of the *in vitro* lipolysis test after 60 min, FFA released and % digested after 60 min of digestion and absolute bioavailability of nilotinib formulations in male Sprague-Dawley rats (mean \pm SD, n = 3, except *in vivo*, where n = 5). The table is sorted according to the *in vivo* absolute bioavailability from high to low.

	FA type ^{a)}	HLB	Nilotinib solubility [mg/mL]	In vitro solubilisation [%] _{b)}	<i>In vivo</i> absolute bioavailability [%]	FFA released [mmol]	% digested
Tween 20	MC	16.7	2.07 ±0.22	1.76 ± 0.11	66.50 ± 21.96	0.63 ± 0.04	75.78 ± 4.25
Labrasol	MC	12	4.12 ± 0.05	0.66 ± 0.07	33.82 ± 11.52	1.69 ± 0.02	91.81 ± 0.98
Labrafil M1944 CS	LC	9	0.88 ± 0.45	0.45 ± 0.08	26.02 ± 6.86	0.99 ± 0.03	33.82 ± 1.04
Span 80	LC	4.3	0.56 ± 0.03	1.12 ± 0.09	25.99 ± 11.48	0.51 ± 0.02	19.09 ± 0.85
Cremophor RH40	LC	15	3.40 ± 0.41	11.07 ± 1.40	22.83 ± 10.00	0.22 ± 0.01	22.61 ± 1.25
Tween 85	LC	11	1.27 ± 0.06	6.40 ± 0.36	19.38 ± 6.89	0.62 ± 0.05	38.61 ± 3.32
Tween 85/ Cremophor RH40 67:33 (w/w)	LC	12.3	1.88 ± 0.08	6.94 ± 0.52	17.08 ± 5.62	0.38 ± 0.01	27.33 ± 0.97
Brij L23	MC	16.9	2.13 ± 0.03	11.80 ± 1.78	16.71 ± 5.27	0.00 ± 0.01	0.00 ± 0.00
Brij O2	LC	4.9	0.69 ± 0.04	0.19 ± 0.05	7.32 ± 3.87	- 0.05 ± 0.02	0.00 ± 0.00

^{a)} MC: medium chain, LC: long chain

^{b)} Aqueous phase concentration in the *in vitro* lipolysis test after 60 min of digestion

387 *Comparing nilotinib solubilisation following in vitro dispersion and digestion.*

A range of surfactant-based formulations containing 10 mg/mL nilotinib were prepared and assessed in the *in vitro* dispersion/lipolysis test. The release and dissolution of nilotinib into the different phases were monitored during dispersion and 60 min of digestion. The concentration of nilotinib in the aqueous phase is shown in Figure 3 and Table 2, the distribution across all phases before initiation of digestion (0), 30 min and 60 min after digestion is shown in Figure S 2 in the supporting information and the FFA released versus time profiles in Figure S 3 in the supporting information.

395



Figure 3. Aqueous phase concentration during 60 min of *in vitro* lipolysis of selected nilotinib suspensions. Cremophor RH40 (\diamond), Brij L23 (*), Tween 20 (\circ), Span 80 (\blacktriangle), Labrasol (\blacksquare), Labrafil M1944 CS (\Box), Tween 85 (\bullet), Brij O2 (×), Tween 85/Cremophor RH40 mixture (67:33 w/w) (\blacktriangledown) (mean ± SD, n = 3).

All formulations displayed good dispersion characteristics upon addition to the media. The highest concentration of nilotinib upon dispersion was observed for Cremophor RH40 with 16 $\pm 1\%$ of the dose dissolved in the aqueous phase. This was followed by Brij L23 with $14 \pm 2\%$ of dissolved nilotinib in the aqueous phase. Approximately 6 - 8% of the nilotinib dose was dissolved in the case of Labrasol, Tween 20, Tween 85 and the Tween 85/Cremophor RH40 mixture, which was mid-range relative to the other tested formulations. Poor solubilisation was observed for Span 80, Brij O2 and Labrafil M1944 CS with concentrations < 1%.

408

409 Upon initiation of digestion Cremophor RH40 and Brij L23 were able to maintain relatively 410 high concentrations for 15 min followed by a decrease in concentration to $11 \pm 1\%$ at 30 min, 411 which was maintained throughout the rest of digestion. The Tween 85/Cremophor RH40 412 mixture and Tween 85 maintained the initial dispersion concentration throughout digestion. 413 Labrasol and Tween 20 showed an initial drop in concentration and a further decrease 414 throughout the 60 min resulting in concentrations below 2%. The surfactants that demonstrated 415 very low nilotinib concentrations < 1% upon dispersion also maintained the low concentrations 416 throughout digestion. The ranking of the nilotinib concentration in the aqueous phase upon 417 dispersion was Cremophor RH40 \geq Brij L23 > Tween 20 \geq Labrasol \geq Tween 85 \geq Tween 418 85/Cremophor RH40 > Span $80 \ge$ Labrafil M1944 CS > Brij O2. After 60 min of digestion the 419 aqueous phase concentration of nilotinib was ranked Brij $L23 \ge Cremophor RH40 > Tween$ 420 85/Cremophor RH40 mixture \geq Tween 85 > Tween 20 \geq Span 80 \geq Labrasol \geq LabrafilM 1944 421 $CS \ge Brij O2$. Overall, there was no apparent relationship between the ranking in solubilisation 422 capacity and surfactant HLB FA chain length or digestibility.

423

In order to gain insights into the nilotinib distribution post-digestion, all samples were separated
into three different phases (solid, aqueous and lipid phase) by centrifugation (Figure S 2). The

426 solid phase represents the undissolved crystalline drug within the surfactant formulation and all 427 formulations showed the highest amount of nilotinib in the solid phase. Additionally, in the 428 case of Labrafil M1944 CS, Span 80 and Brij O2 an oily phase was detected throughout 429 digestion. In all cases the concentration of nilotinib increased in the oily phase as digestion 430 progressed.

431

432 *Ranking the in vivo bioavailability of surfactants*

The *in vivo* performance of nilotinib surfactant formulations was evaluated in male Sprague-Dawley rats. An aqueous suspension containing 0.5% (w/v) methylcellulose (for stabilisation) was used as a non-surfactant control formulation, as previously described. ⁴⁶ The absolute bioavailability is shown in Figure 4, the plasma concentration versus time profiles are presented in Figure S 4 in the supporting information and the pharmacokinetic parameters in Table 3.



440 Figure 4. Absolute bioavailability (F_{abs}) of nilotinib suspensions in male Sprague-Dawley rats
441 after oral administration of 20 mg/kg nilotinib and 2 mL/kg excipient in comparison to an
442 aqueous suspension, as previously described. ⁴⁶ Tween 20 is significantly different to all other
443 tested surfactants and the aqueous suspension. Tween/Cremophor is a mixture of Tween 85 and
Page 23

- 444 Cremophor RH40 (67:33 w/w). Shaded area represents long chain excipients and non-shaded
- 445 area medium chain excipients (mean \pm SD, n = 5).
- 446
- 447

448 **Table 3.** Pharmacokinetic parameters of nilotinib after oral administration of 20 mg/kg nilotinib and 2 mL/kg excipient to male Spraque-Dawley rats.

449 Nilotinib was administered as surfactant suspensions (n = 5). t_{max} , mean residence time (MRT) and mean absorption time (MAT) are given as median

450	(range), all other paramet	ters as mean ± SD.
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	Pharmacokinetic parameters						
	AUC 0 h – inf. $[\mu g^{*}h/mL]$	c _{max} [µg/mL]	<i>t</i> _{max} [h]	MRT [h]	MAT [h]	$F_{abs} \left[\%\right]{}^{b)}$	
Aqu. susp. ^{a)}	14.33 ± 4.24	2.65 ± 0.68	2 (2-4)	4.14 (3.34 - 4.83)	2.49 (1.69 - 3.18)	12.90 ± 3.34	
Tween 20	73.89 ± 24.40	10.28 ± 3.64	2 (2-6)	5.59 (4.97-6.96)	3.94 (3.32-5.32)	66.50 ± 21.96	
Tween 85	21.54 ± 7.66	3.13 ± 0.84	4 (2-4)	6.05 (4.56-6.29)	4.40 (2.91-4.64)	19.38 ± 6.89	
Labrasol	37.59 ±12.80	4.76 ± 1.83	10 (6-10)	7.62 (6.35-8.00)	5.97 (4.70-6.35)	33.82 ± 11.52	
Labrafil M1944 CS	28.91 ±7.63	4.18 ± 1.00	8 (4-10)	7.50 (6.03-8.50)	5.86 (4.38-6.85)	26.02 ± 6.86	
Cremophor RH40	25.37 ± 11.11	3.43 ± 1.40	10 (2-10)	7.56 (4.46-7.65)	5.92 (2.82-6.00)	22.83 ± 10.00	
Span 80	28.88 ± 12.75	4.30 ± 2.23	8 (8-10)	7.88 (6.65-8.41)	6.23 (5.00-6.77)	25.99 ± 11.48	
Brij O2	8.14 ± 4.30	0.98 ± 0.38	4 (1-8)	5.99 (4.58-6.76)	4.35 (2.94-5.12)	7.32 ± 3.87	

Page 25

Brij L23	18.57 ± 5.85	2.77 ± 1.31	10 (6-10)	7.50 (7.26-8.31)	5.85 (5.61-6.67)	16.71 ± 5.27
Tween 85:Cremophor RH40 (67:33 w/w)	18.98 ± 6.24	2.93 ± 1.01	4 (2-8)	5.70 (5.43-7.49)	4.05 (3.78-5.84)	17.08 ± 5.62

451 ^{a)} Data as previously described by Koehl *et al.* ⁴⁶

452 ^{b)} Intravenous data obtained from Koehl *et al.* ⁴⁶

453 Interestingly, the highest exposure was observed for the medium chain surfactant Tween 20 454 with an absolute nilotinib bioavailability of $66.5 \pm 22.0\%$. This was statistically significant 455 higher compared to all other study arms and the aqueous suspension (p < 0.05). The Labrasol 456 formulation increased bioavailability to $33.8 \pm 11.5\%$, which was significantly higher relative 457 to the Brij O2, a non-digestible formulation. While there was a trend towards an increased 458 bioavailability for the Labrasol formulation compared to the aqueous suspension, the increases 459 did not reach statistical significance. In contrast, the Brij O2 formulation showed a trend 460 towards a decreased bioavailability of $7.3 \pm 3.9\%$ compared to the aqueous suspension, 461 although not statistically significant. All other formulations (Brij L23, Tween 85/Cremophor 462 RH40 mixture, Tween 85, Cremophor RH40, Span 80, Labrafil M1944 CS and the aqueous 463 suspension) displayed similar *in vivo* bioavailability of between $16.7 \pm 5.3\%$ to $26.0 \pm 6.9\%$.

464

465 *Relationship between surfactant digestibility and in vivo bioavailability*

466 The surfactant properties were compared to the absolute bioavailability obtained in the *in vivo* 467 study. Surfactants with different HLB values resulted in a similar absolute bioavailability. 468 Therefore, no relationship between the *in vivo* performance and the HLB value was established. 469 While the two top performing surfactants contain medium chain FA, the third lowest absolute 470 bioavailability was observed for Brij L23, also containing medium chain FA. In the case of the 471 digestion independent Brij L23, however, the FA was not released. The results, therefore, 472 indicate that in terms of released FA during digestion the in vivo performance might be related 473 to FA chain length. The most promising in vitro-in vivo-relationship was observed between 474 surfactant digestibility and the in vivo bioavailability. Figure 5 displays the absolute 475 bioavailability versus % digested and Figure S 5 in the supporting information shows absolute 476 bioavailability versus FFA released. The strongest trend was observed between the absolute bioavailability and the % surfactant digested ($r^2 = 0.5628$). In the case of the *in vitro-in vivo-*477

478 relationship for the absolute bioavailability versus FFA released the overall trend was poor 479 $(r^2 = 0.1811)$, which may reflect that overall the extent of surfactant digestion is more reliable 480 representing the *in vivo* performance than the amount of FFA released.

481





483Figure 5. Absolute bioavailability (Fabs) versus % digested of Cremophor RH40 (\diamond), Brij L23484(*), Tween 20 (\circ), Span 80 (\blacktriangle), Labrasol (\blacksquare), Labrafil M1944 CS (\Box), Tween 85 (\bullet), Brij O2485(\times), Tween 85/Cremophor RH40 mixture (67:33 w/w) (\blacktriangledown) (mean ± SD, Fabs n = 5, % digested486n = 3).

488 DISCUSSION

489 Selection of surfactants during oral formulation development requires a in depth understanding 490 of the surfactant characteristics both in vitro and in vivo. Non-ionic surfactants such as Tweens 491 and Poloxamers are widely used excipients in commercial formulations to facilitate a higher 492 dissolution rate and improve solubility. The solubilising effects are typically influenced by digestion and surface-active excipients can affect digestion itself. ^{51, 52} Surfactants may further 493 affect drug permeation, drug efflux and potentially metabolism in enterocytes. ^{12, 13, 16, 21} 494 495 Surfactant selection in oral formulation is largely empirically driven, with limited consideration 496 of the impact of surfactant properties or likely surfactant digestion on the *in vivo* performance. 497 This study, therefore, addresses the need for a systematic comparison of surfactant digestibility 498 and assessment of surfactant properties on *in vivo* performance using nilotinib as a model poorly 499 water-soluble compound.

500

501 To the best of our knowledge, this is the first study to compare digestibility of a range of 15 502 commonly used surfactants and hence this allows the establishment of a digestibility databank 503 for surfactants. Overall, the findings suggested that surfactants containing medium chain FAs 504 displayed a higher digestibility compared to surfactants containing long chain FAs. This finding agrees with previous reports of smaller sets of surfactants ⁴⁹ as well as glycerides ⁵³ and lipid 505 formulations. ⁴³ The lower digestibility of surfactants containing long chain FAs can be 506 attributed to the extent of long chain FA ionisation ^{43, 54} and the limited solubilisation capacity 507 508 of the digestion media for the long chain FAs. ^{43, 53} Long chain FAs are ionised to a lower extent relative to medium chain FAs at the pH of 6.5. ^{43, 53} Therefore, the non-ionised long chain FAs 509 510 are in need of micellar solubilisation due to a poor solubility in the digestion media. Once the 511 solubilisation capacity of the digestion media for long chain FAs is reached, the long chain FAs accumulate at the droplet/micellar interface, effectively inhibiting further enzyme binding and 512

digestion. ⁴³ In addition, the analysis of the surfactant digestibility revealed that digestibility 513 514 was not influenced by the HLB value. This indicates that surfactant digestibility was not readily 515 predictable and highlights the importance of *in vitro* lipolysis in the characterisation and selection of surfactants in oral dosage forms. It should also be acknowledged that the reported 516 517 in vitro digestibility (FFA released and % digested) might not be entirely translatable to an in 518 vivo digestibility, as non-ionic surfactants and their lipolytic products can inhibit digestion in 519 vitro depending on the given conditions. For example, Cremophor RH40, Cremophor EL, Tween 80, Tween 20 and Brij L23 are known to inhibit digestion to varying extents ^{51, 55, 56} 520 leading to a lower or slower ⁵⁷ digestion compared to *in vivo*, where the inhibiting surfactants 521 522 and their lipolytic products could be absorbed. Additionally, the type of enzyme or enzymatic 523 extract as well as pH of the media has been shown to influence the enzymatic activity and consequently the overall extent of digestion, as shown for the excipient Labrasol. ⁵² Thus, the 524 525 total amount of released FFA and/or a given % digested depends on the employed in vitro or in 526 *vivo* system.

527

528 Among the surfactants, the highest absolute bioavailability was observed for the medium chain 529 excipients Tween 20 (F_{abs} 66.50 ± 21.96%), which was statistically significant higher compared 530 to all other surfactants (p < 0.05). The second highest bioavailability was observed for the medium chain surfactant Labrasol, which was statistically significant higher compared to Brij 531 O2 (p < 0.05). Both Tween 20 and Labrasol are digestible medium chain-based surfactants 532 533 indicating that the *in vivo* performance might be influenced by the release of medium chain FA. 534 In terms of the HLB value, a relationship to the in vivo performance was not apparent. 535 Additionally, the *in vivo* performance was not influenced by the solubility of nilotinib in the 536 excipients. For example, even though the dose-solubility ratio for Brij L23 (4.7) and Tween 20 537 (4.8) were similar, the bioavailability was significantly higher for the Tween 20 formulation. 538 This suggests that the solubility in the surfactants was not a limiting factor to the oral 539 bioavailability.

540

Interestingly, the study suggested that the *in vivo* exposure was influenced by the digestibility 541 542 of the surfactants, and in general highly digestible surfactants such as Labrasol, Tween 20 and 543 Labrafil M1944 CS displayed the highest bioavailability. Similarly, the two least digested 544 surfactants Brij L23 and Brij O2 showed the lowest bioavailability, which may suggest that the 545 drug was trapped within the non-digestible surfactant micelles. This finding is in line with the 546 observation by Berthelsen and co-workers where bioavailability did not increase with 547 increasing surfactant concentration due to an entrapment in Cremophor RH40 micelles (which 548 displayed lower digestibility relative to Cremophor EL). In contrast, in the case of the 549 Cremophor EL surfactant micelles, which displayed higher digestibility, bioavailability increased with increasing surfactant concentration. ⁵⁰ It should also be noted that it has been 550 551 reported in the literature that surfactant only formulations of either Cremophor RH40 or EL did not lead to significant differences in bioavailability of danazol in dogs.⁴⁹ On the contrary, when 552 553 incorporating surfactants into self-emulsifying lipid-based formulations, the poorly digestible 554 surfactant Cremophor RH40 (55% w/w) displayed higher oral bioavailability of danazol 555 compared to Cremophor EL (55% w/w). Possible reasons for this differing outcome between 556 studies, may reflect the differing role of surfactant between different formulation types. Specifically, in the case of self-emulsifying systems, the surfactants also serve to support self-557 558 emulsification and stabilisation of the emulsified oil phase. As such, digestion of surfactants 559 may lead to destabilisation of the emulsion droplet, leading to drug precipitation. Therefore, for 560 oil containing self-emulsifying formulations, it appears that low digestibility surfactants are 561 favoured to improve emulsion stability during digestion and reduce the risk of drug precipitation from the oil droplets during lipolysis. However, our study confirmed that in the 562

563 case of nilotinib surfactant only systems, low digestibility surfactants may have led to lower

overall in vivo exposure most likely via entrapment within surfactant micelles.

565

564

566 Compared to the aqueous suspension, Tween 20 was the only surfactant that showed a 567 statistically significant higher bioavailability. All other surfactants displayed a bioavailability 568 between approximately 7.3% and 33.8%, which compared favourably to a previous report of nilotinib bioavailability of 34% in rats using a cremophor based micellar solution. ³⁵ One 569 570 explanation of the impressive in vivo performance of Tween 20 may be additional 571 biopharmaceutical benefits of this excipient. In vitro cell assays have shown that Tween 20 is 572 a P-gp (or MDR-1) inhibitor, ^{15, 16} reducing the efflux into the intestinal lumen. In the case of nilotinib transport by P-gp was demonstrated with an efflux ratio (basolateral/apical) of 3.9-4.1 573 using Caco-2 cells. ^{35, 37, 38} This indicated that further P-gp inhibition by formulation excipient 574 575 such as Tween 20 could have contributed positively to nilotinib's bioavailability. A study by 576 Cornaire *et al.* showed that the effectiveness to inhibit P-gp transport for digoxin was Labrasol > 577 Imwitor 742 > Acconon E = Softigen 767 > Cremophor EL > Miglyol > Solutol HS 15 > Sucrose monolaurate > Tween 20 > TPGS > Tween 80 using the rat everted gut sac model. ⁵⁸ 578 579 In addition, it was shown that Labrafil M1944 CS was not active as transport enhancer for digoxin. ⁵⁸ P-gp as well as CYP3A4 share a significant overlap in substrate specificity, ⁵⁹ which 580 581 is also the case for nilotinib, which is mainly metabolised by CYP 3A4 in the enterocytes and liver.³⁵ In fact, co-administering nilotinib with grapefruit juice (intestinal CYP enzyme 582 inhibitor) increased the AUC by 29% ³⁹ showing a significant pre-systemic clearance. A study 583 584 by Ren and co-workers showed that Tween 20 is a strong CYP 3A4 inhibitor using rat liver and 585 intestinal microsomes. In comparison to the four tested non-ionic surfactants (Tween 20, 586 Cremophor EL, Myrj S40 and Pluronic F68), Tween 20 was the most potent inhibitor. Additionally, the study confirmed a significant higher AUC for diazepam when co-587

administered with Tween 20 as well as a decrease in the metabolite (1-hydroxymidazolam) AUC to about 40%. ²¹ Therefore, the impact of Tween 20 on CYP 3A4 was more distinctive relative to P-gp and overall this would tend to suggest that the effect of CYP 3A4 is more relevant in the case of nilotinib, as also suggested by the significant increased bioavailability in the presence of grapefruit juice. ³⁹

593

594 During digestion FFAs are released, which generate a variety of colloidal species in 595 combination with bile salts, phospholipids and lipolytic products, which potentially have a 596 higher solubilisation capacity for drugs. For nilotinib it was previously shown that the solubility 597 in post-digestive media of a lipid formulation was increased.²⁴ In addition, it was reported that the solubility of nilotinib is influenced by the bile salt concentration as evidenced by a higher 598 solubility in FeSSIF (3.2 \pm 0.1 µg/mL) compared to FaSSIF (0.3 \pm 0.03 µg/mL). ²⁴ As FFAs 599 600 can increase the bile salt release *in vivo*, ⁶⁰⁻⁶² digestion appears to be a crucial parameter for the 601 success of the surfactant-only formulations. This was especially apparent for the digestible 602 Tween 20 and non-digestible Brij L23, which both contain the medium chain FA lauric acid. The non-digestible formulation resulted in a significantly lower exposure indicating that the 603 604 release of lauric acid may have been the driving factor for an improved bioavailability, as FFAs 605 have also shown beneficial effects on drug permeability without pronounced cytotoxic effects. ^{25, 28-30, 32, 33, 63-65} As nilotinib is passively transported, ⁶⁶ the permeability enhancing effects of 606 surfactants ¹² and their lipolytic products may have contributed to a higher bioavailability of 607 608 nilotinib when compared to the aqueous suspension.

609

610 The *in vitro* lipolysis of Brij L23 and Cremophor RH40 formulations showed high 611 concentrations of solubilised nilotinib in the aqueous phase upon dispersion and throughout 612 digestion. For both surfactants a drop in nilotinib concentrations of approximately 4.9% after

15 min of digestion was observed. Similarly, the nilotinib aqueous phase concentration 613 614 decreased in the case of Labrasol and Tween 20 upon initiation of digestion resulting in a total 615 decrease of approximately 6.2% and 6.8%, respectively. However, the drop in nilotinib 616 concentration in the aqueous phase did not seem to be exclusively related to the digestibility of 617 these surfactants (Figure 1), as Labrasol and Tween 20 are highly digested, but Cremophor 618 RH40 and Brij L23 are poorly or not digested. Furthermore, Labrafil M1944 CS and Tween 85, 619 which are both rapidly and > 33% digested (Figure S 3, Figure 1), did not show a decrease in 620 nilotinib concentration upon initiation of digestion. However, given that Tween 20 and Labrasol 621 are both medium chain-based surfactants, it appears that the initial drop in nilotinib aqueous 622 phase concentration upon initiation of digestion most likely reflects the combination of high 623 digestibility and the release of medium chain FAs. This may reflect the lower solubilisation capacity of medium chain post digestive media, as previously reported. ^{67, 68} While there was 624 625 no direct relationship between the HLB value of the surfactants and the performance in the *in* 626 *vitro* lipolysis, there seemed to be a trend that surfactants with a HLB value > 10 performed 627 better compared to surfactants with a HLB value < 10. For example, Brij L23 (HLB value: 16.9) 628 showed an *in vitro* solubilisation of $11.8 \pm 1.8\%$, whereas Brij O2 (HLB value: 4.9) only 629 reached an *in vitro* solubilisation of $0.2 \pm 0.1\%$. Surfactants with a HLB value > 10 are 630 considered suitable for stabilising oil in water systems, whereas surfactants with a HLB value < 10 are suitable for stabilising water in oil systems. Thus, the higher HLB surfactants seem to 631 632 stabilise solubilised nilotinib, which is highly lipophilic ($\log P$: 4.95), in the aqueous phase better compared to low HLB surfactants. In contrast to the HLB value trend, the in vitro 633 634 lipolysis test indicated that digestibility of the surfactants is not related to nilotinib solubilisation 635 in the aqueous phase in vitro. For example, Brij L23 (non-digestible) and Cremophor RH40 636 (digestible) demonstrated comparable aqueous phase concentrations but different digestibility. 637 Additionally, no trend was established between the FA chain length and the aqueous phase

concentration of nilotinib. The aqueous phase concentration of the *in vitro* lipolysis is thought 638 639 to represent the amount of drug readily available for absorption *in vivo* and is commonly used 640 to rank formulation performances. However, due to the limitations of the *in vitro* systems, such 641 as the lack of an absorptive sink, low media volume in combination with a high drug and 642 excipient load as well as different hydrodynamics and enzyme activity compared to in vivo, a relationship between *in vitro* and *in vivo* performance for surfactants ^{49, 50} or other formulations 643 ^{24, 69, 70} has been reported difficult in some cases. Also, this study could not demonstrate a 644 645 correlation between the aqueous phase concentration of the *in vitro* lipolysis test and the *in vivo* 646 performance. For example, the relatively higher in vitro solubilisation of Brij L23 and 647 Cremophor RH40 did not correlate with a higher *in vivo* performance, as bioavailability values 648 of Cremophor RH40 and Brij L 23 were in the mid (approximately 23%) and low (approximately 17%) end of the range of absolute bioavailability obtained in vivo. An 649 650 adjustment of the in vitro model to rat conditions (e.g. low enzyme activity and low gastrointestinal volumes), ^{71, 72} two stages (gastric and intestinal) ⁷³⁻⁷⁵ or the addition of an 651 absorptive sink ^{76, 77} may offer additional insights into the *in vivo* behaviour. 652

653

654 CONCLUSION

655 The present study systematically investigated the relationship between non-ionic surfactant properties and the *in vitro* and *in vivo* performance using nilotinib. Tween 20 demonstrated an 656 impressive 5.2-fold increase in absolute bioavailability when compared to an aqueous 657 suspension. In general, surfactants that displayed high digestibility in vitro displayed higher 658 659 nilotinib bioavailability in vivo. Medium chain FA-based surfactants appeared to be favourable 660 to increase bioavailability compared to long chain FA types. However, HLB of the surfactant did not correlate with the in vivo performance of nilotinib. The reported additional 661 biopharmaceutical effects of Tween 20, in terms of inhibition of CYP 3A4 and modulation of 662

P-gp efflux, may explain the impressive increase in bioavailability. Therefore, this study highlights the importance of appropriate surfactant selection to maximise *in vivo* exposure, with careful consideration of solubilisation properties, impact of digestion and biopharmaceutical effects. Further studies using broader range of drugs are therefore merited with the ultimate aim of developing a bio-predictive surfactant classification system.

668

669 SUPPORTING INFORMATION

Nilotinib solubility in studied excipients, distribution of nilotinib into different phases during *in vitro* lipolysis, FFA released versus time profiles during *in vitro* lipolysis, plasma
concentration versus time profiles of nilotinib in rats, relationship of F_{abs} and FFA released.

673

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