Postprandial administration but not controlled release in the colon increases oral bioavailability of DF030263, a promising drug candidate for chronic lymphocytic leukemia

Jong Bong Lee[a], Xiaowei Zang[b], Atheer Zgair[a,c], Ting Qian Ooi[a], David W Foley[a] , Gregory Voronin[d], Leonid Kagan[b], Fadi Soukarieh[a], Rui Gao[a], Hao Shao[a,e], Wan Tying Soh[a], Tae Hwan Kim[f], Min Gi Kim[f], Hwi-yeol Yun[g], Anthony J Wilson[a], Peter M Fischer[a], Pavel Gershkovich[a*]

[a] School of Pharmacy & Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK

[b] Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

[c] College of Pharmacy, University of Anbar, Anbar, Iraq

[d] Comparative Medicine Resources, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

[e] Hunan Key Laboratory of Molecular Precision Medicine, Department of Oncology, Xiangya Hospital, Central South University, Changsha, Hunan, China

[f] School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea

[g] College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea

***Corresponding author:** Pavel Gershkovich, PhD
School of Pharmacy, Centre for Biomolecular Sciences
University of Nottingham, University Park
Nottingham, UK
NG7 2RD
Tel: +44 (0) 115 846 8014
Fax: +44 (0) 115 951 3412
Email: pavel.gershkovich@nottingham.ac.uk

ABSTRACT

For treatment of chronic cancers, the oral administration route is preferred as it provides numerous advantages over other delivery routes. However, these benefits of oral chemotherapy can be limited due to unfavorable pharmacokinetics. Accordingly, pharmacokinetic development of chemotherapeutic agents is crucial to the improvement of cancer treatment. In this study, assessment and optimization of biopharmaceutical properties of a promising drug candidate for cyclin-dependent kinase 9 (CDK9) inhibitor (DF030263) was performed to promote oral delivery. Oral bioavailability of DF030263 in fasted rats was 23.8%, and a distinct double-peak phenomenon was observed. A two-site absorption windows mechanism was proposed as a possible explanation to the phenomenon. The two-site absorption window hypothesis was supported by *in vitro* solubility assays in biorelevant fluids with different pH levels, as well as by *in silico* simulation by GastroPlus™. Controlled release to the colon was conducted in rats in order to exploit the colonic absorption window but did not improve the oral bioavailability. On the other hand, oral administration at postprandial conditions in rats (performed based on the high *in vitro* solubility in fed state simulated fluid and reduced pH-dependency) resulted in an almost 3-fold increase in bioavailability to 63.6%. In conclusion, this study demonstrates an efficient *in vitro-in vivo-in silico* drug development approach for improving the oral bioavailability of DF030263, a promising candidate for the treatment of chronic lymphocytic leukemia.

1. Introduction

Treatment of cancer is often limited by lack of efficacy and serious adverse effects. Unsatisfactory efficacy in many cases is related to unfavorable pharmacokinetics and biodistribution profiles including poor bioavailability, rapid clearance and limited distribution to the tumor tissues [1]. The key aspect of this is that sufficient drug concentration has to be achieved and maintained at the site of action for the optimal anticancer efficacy [2]. Therefore, along with the development of targeted anticancer therapies by means of well-defined molecular targets or biologic signaling streams, pharmacokinetic development and optimization of chemotherapeutic agents are important to improve the treatment outcomes of cancer [3].

While the majority of chemotherapy regimens in cancer depends on parenteral delivery, oral administration of anticancer agents is desired for several reasons. Oral dosing allows self-administration by the patients and avoids the inconvenience of intravenous injections which needs hospitalization [4, 5]. Therefore, it improves patient compliance and reduces costs of therapy. It also prevents risk of infection that might be caused as a complication of injectable routes of administration. Most importantly, it makes treatment of chronic diseases more practical [5, 6]. However, many anticancer agents possess unfavorable properties that make sufficient systemic exposure following oral administration challenging. These properties are mainly physicochemical characteristics that result in low solubility, poor permeability, high efflux and rapid pre-systemic metabolism [2].

Cyclin-dependent kinases (CDKs) are essential in cell growth as they control progression of cell cycles and regulate transcription [7]. CDK inhibitors have been discovered and developed on this premise to seek inhibition of unsuppressed cancer cell proliferation [7, 8]. Among the

CDK family, CDK9 is particularly related to the regulation of RNA transcription. High expression of CDK9 and cyclin T1, corresponding cyclin partner of CDK9, was observed in chronic lymphocytic leukemia (CLL), indicating their key roles in pathologic mechanism of the disease [8-10]. We have recently reported a series of highly active and selective inhibitors of CDK9 as candidates for the treatment of CLL. Among the candidates, DF030263 was one of the most efficacious and selective compounds *in vitro* [11].

Therefore, the aim of this work was to assess and to optimize the biopharmaceutical properties of DF030263 in order to achieve efficient treatment of CLL following oral administration of this compound. An *in vitro-in vivo-in silico* approach was applied to provide adequate solution towards improvement of the oral bioavailability. Biopharmaceutical optimization approaches including controlled release in the colon and postprandial conditions have also been assessed in this work.

2. Material and methods

2.1. Materials

DF030263 (5-(2-((3-(1,4-diazabicyclo[3.2.2]nonan-4-yl)phenyl)amino)-5-methylpyrimidin-4-yl)-N,4-dimethylthiazol-2-amine, Figure 1) was synthesized in School of Pharmacy, University of Nottingham (Nottingham, UK) as reported as compound **30m** in our previous publication [11]. Sodium taurocholate (NaTc), NaCl, NaOH (pellets), NaH_2PO_4, glacial acetic acid, lecithin, chlorpromazine and dexamethasone were obtained from Sigma (Gillingham, UK). Rat plasma was purchased from Sera Laboratories International (West Sussex, UK). Polyethylene glycol (PEG) 400 and all solvents (HPLC grade or higher) were obtained from Fisher Scientific (Leicestershire, UK). All other chemicals were analytical reagent grade or higher.

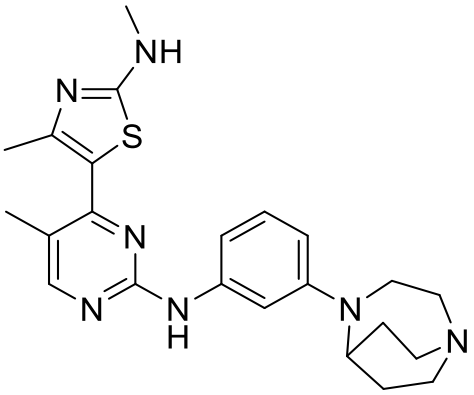


Figure 1. Chemical structure of DF030263 [11].

2.2. *In vitro* solubility assay

2.2.1. Preparation of simulated fluids

Three different types of fluids simulating the gastrointestinal environment were prepared according to previously reported preparation methods [12-14]: fasted state simulated gastric fluid (FaSSGF), fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF). The composition of these three simulated fluids were as described in Table 1. Prior to the assay, the pH of FaSSGF was adjusted to 1.6 and 3.0 using HCl and the pH of FaSSIF and FeSSIF was adjusted to be between 5.0-7.8 with interval of 0.2 using HCl or NaOH. All fluids were prepared on the day before the assay and stored at 4°C until use.

Table 1. Composition of the simulated fluids (mM)

	FaSSGF	**FaSSIF**	**FeSSIF**
NaTc	0.08	3	15
Lecithin	0.02	0.75	3.75
NaCl	34.2	105.9	203.3
NaOH	-	8.7	102
NaH_2PO_4	-	8.2	-
Acetic acid	-	-	144

FaSSGF, fasted state simulated gastric fluid; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid.

2.2.2. Solubility assay

On the day of assay, 198 µL of test medium (FaSSGF, FaSSIF, FeSSIF and water) was aliquoted into centrifugal tubes (Costar Spin-X Centrifuge Tube, Fisher Scientific, Leicestershire, UK). A volume of 2 µL of 20 mM stock solution in DMSO was then spiked into each tube to yield 200 µM of test concentration. The tubes were incubated at 37°C shaking at 250 rpm for 2 h using a shaking incubator (Thermo Scientific MaxQ4000, Thermo Scientific, OH, USA). After the incubation, the samples were immediately centrifuged for 5 min at 2400 *g* (Heraeus Fresco 17 Centrifuge, Thermo Electron, MA, USA). The filtrate was collected and subjected to analysis as described below. The assay was performed in triplicate.

2.2.3. Sample analysis

To 50 µL of sample, 10 µL of internal standard stock solution (100 µM dexamethasone, 50% acetonitrile in water) was spiked. For FaSSGF samples, 200 µL of 1 M NaOH was added and for FaSSIF, FeSSIF and water samples, 200 µL of 0.1 M NaOH was added. Two mL of methyl-tert-butyl ether was then added and the mixture was vortex-mixed for 10 min and centrifuged at 1160 *g* for 10 min. The organic layer was transferred and evaporated to dryness under N_2 gas at 40°C. Hundred µL of 40% acetonitrile in water was then added for reconstitution and vortex-mixed for 10 min before being transferred to HPLC vial for analysis.

The prepared samples were analyzed by a HPLC-UV system consisting of a Waters 600 Pump, Waters 717 Autosampler and Waters 2996 Photodiode Array Detector. A separate column oven was used to maintain the column temperature at 40°C. The stationary phase was a Gemini C18 250 × 4.6 mm, 5 µm particle size equipped with a SecurityGuard 2 × 4 mm, 3 µm particle size (Phenomenex, Macclesfield, UK). Mobile phase was a mixture of acetonitrile and 10 mM ammonium acetate buffer with pH adjusted to 5.0 with glacial acetic acid (40:60, v/v). The

flow rate was 0.5 mL/min and 60 µL was injected. Chromatograms were observed at 256.5 nm of UV wavelength.

2.3. *In vivo* pharmacokinetic experiment

2.3.1. Animals

This study was conducted in accordance with an approved protocol by the Institutional Animal Use and Care Committee at Rutgers, The State University of New Jersey (#16-001). Male Sprague Dawley rats (Envigo, Inc., Indianapolis, IN) weighing 300-350 g were used for the experiment. Animals were housed at controlled temperature, 12 h light/dark cycle and with free access to food and water. The acclimatization of the animals was at least for four days. Surgical procedures were performed under general anesthesia induced by inhalation of isoflurane with an air carrier (3% for induction and <3% for maintenance). All rats underwent right jugular vein cannulation for blood sampling. For the group that received colonic administration, cannulation of the cecum was performed based on a previously reported protocol [15]. Laparotomy was performed to gain access to the large intestine. A small hole in the cecum was made with a 19 G needle and the cannula (built using PE-50 tubing) was inserted. Animals were allowed to recover for two days and were fasted up to 12 h before the pharmacokinetic experiment (except postprandial conditions experiment) with free access to drinking water.

2.3.2. Pharmacokinetic experiments

Formulations of DF030263 were prepared in PEG400:water (50:50, v/v) in concentrations of 2 or 4 mg/mL. For intravenous administration, formulation of 2 mg/mL was delivered at 1 mL/kg via the jugular vein cannula followed by 0.3 mL of heparinized saline (50 IU/mL) to ensure complete administration. Oral administration was conducted with the formulation of 4 mg/mL at 3 mL/kg using an oral gavage tube. Colonic administration was also performed with

the formulation of 4 mg/mL at 3 mL/kg delivered via the cannula inserted into the cecum. The formulation was infused using a syringe pump (PHD Ultra, Harvard Apparatus, Holliston, MA, USA) at a constant rate for 1 h for colonic administration. Following the administration, blood samples (250 µL) were collected from the jugular vein cannula at pre-determined time points. Blood samples were centrifuged (3000 *g*, 10 min) and plasma samples were stored in -80°C until analysis.

2.3.3. Sample analysis

A volume of 100 µL of plasma was used for sample preparation procedure. The plasma samples were spiked with 10 µL of internal standard stock solution (5 µg/mL chlorpromazine, 50% acetonitrile in water). Three hundred µL of 0.1 M NaOH and 2 mL of methyl-tert-butyl ether were then added. The mixture was vortex-mixed for 1 min and centrifuged at 1160 *g* for 10 min. The supernatant organic layer was transferred to new glass tubes and evaporated to dryness under gentle stream of N_2 gas at 40°C. Hundred µL of 40% acetonitrile in water was added to dried samples for reconstitution and vortex-mixed for 30 s before being transferred to HPLC vial for analysis.

The HPLC-UV system was composed of Agilent 1260 Infinity equipped with quaternary pump, high performance autosampler, thermostated column compartment and diode array detector. The stationary phase was a Gemini C18 250 × 4.6 mm, 5 µm particle size equipped with a SecurityGuard 2 × 4 mm, 3 µm particle size (Phenomenex, Macclesfield, UK) and the column temperature was maintained at 40°C. Mobile phase was a mixture of acetonitrile, methanol and 10 mM ammonium acetate buffer with pH adjusted to 5.0 with glacial acetic acid (30:20:50, v/v). The flow rate was 0.4 mL/min and 80 µL was injected. Chromatograms were observed at 256.5 nm of UV wavelength.

Sample stability was tested prior to the pharmacokinetic experiment to ensure sample integrity during sample storage and analysis. Samples of rat plasma (n = 4) spiked with low (25 ng/mL) and high (8 μg/mL) quality control concentrations were prepared and stored at the following conditions: 4 h at room temperature to test bench-top stability; 1, 2 and 4 weeks at -80°C to test storage condition stability. Autosampler stability was tested at the same concentrations with processed samples stored at 5°C for 24 h. All stability results were within ±15% relative error. Accordingly, all sample storage and preparation were performed within the limit of stability tested.

2.4. Deconvolution of plasma concentration-time profiles

Deconvolution of plasma concentration-time profiles was conducted using Phoenix WinNonlin version 6.3 (Certara, Princeton, NJ, USA). The mean plasma concentration-time profiles following oral and intravenous administrations were used for the deconvolution. The results of deconvolution were expressed as cumulative input vs time and input rate vs time.

2.5. *In silico* simulation of absorption sites

Intestinal absorption of DF030263 compound in different compartments of the gastrointestinal tract was simulated *in silico* using GastroPlus™ version 9.0.0007 with built-in ADMET Predictor™ version 7.2.0.0 module (Simulations Plus, Inc., Lancaster, CA, USA). The input parameters are listed in Table 2. Parameters and settings not mentioned in the Table 2 were used as predicted by the ADMET Predictor™ or as given by software default. The physiology setting was selected as “Rat-Fasted” or “Rat-Fed” and paracellular permeation option was turned on. Percentages of the dose absorbed at different compartments of the gastrointestinal tract were simulated at both fasted and fed states for rats.

Table 2. Input parameters for *in silico* simulation of intestinal absorption of DF030263 compound using GastroPlus™

Parameters	Value
Molecular weight (g/mol)	435.6
Log P	3.1
pK_a	10.7
Human jejunal permeability ($\times 10^{-4}$ cm/s)	1.37[a]
Diffusion coefficient ($\times 10^{-5}$ cm^2/s)	0.6288[a]
Drug particle density (g/mL)	1.2[b]
Mean precipitation time (sec)	900[b]
Reference solubility (mg/mL)	0.03846[c]
Biorelevant solubilities (mg/mL)	
FaSSGF (pH 1.6)	0.6737[c]
FaSSIF (pH 6.6)	0.04935[c]
FeSSIF (pH 5.0)	0.06529[c]

[a] Predicted by GastroPlus™
[b] GastroPlus™ default values
[c] Experimental results

2.6. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical significance of differences between two groups was determined by two-tailed unpaired t-test. A *p*-value of less than 0.05 was defined as statistically significant. Statistical analysis was performed using GraphPad Prism version 7.01 (GraphPad Software, Inc., La Jolla, CA, USA). Plasma pharmacokinetic parameters were obtained by non-compartmental analysis using Phoenix WinNonlin 6.3 software (Certara, Princeton, NJ, USA).

3. Results

3.1. *In vivo* oral bioavailability evaluation of DF030263

In vivo oral bioavailability of DF030263 was evaluated in rats and the mean plasma concentration-time profiles are shown in Figure 2. The plasma concentration-time profile obtained following oral administration showed a distinct double-peak phenomenon, which was not apparent following intravenous administration. The first peak appeared after rapid absorption of the compound at 0.5 h while the second peak followed a delayed absorption, appearing at 4 h. Although the time between the two peaks varied for individual animals, this double-peak phenomenon was observed in all rats tested for oral administration (n = 4). The mean oral bioavailability of DF030263 was determined to be 23.8% and other pharmacokinetic parameters obtained from the plasma concentration-time profiles are shown in Table 3.

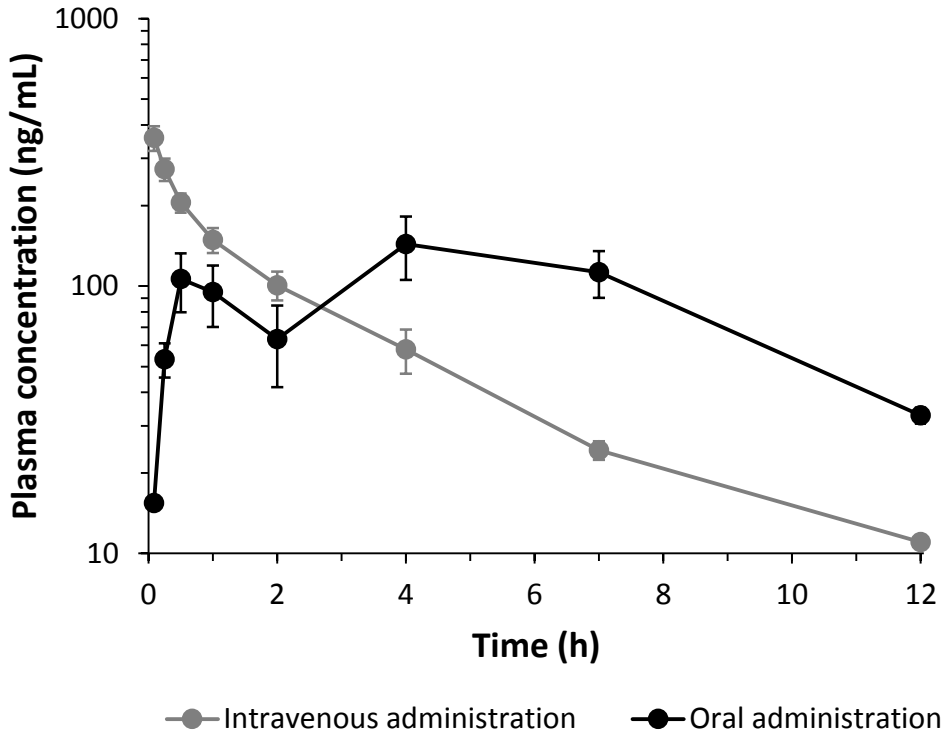


Figure 2. Mean plasma concentration-time profiles of DF030263 in rats following intravenous (2 mg/kg) and oral (12 mg/kg) administration (n = 4, each group).

Table 3. Plasma pharmacokinetic parameters obtained from *in vivo* pharmacokinetic experiments

Parameters	**Intravenous administration (n = 4)**	**Oral administration at fasted state (n = 4)**	**Oral administration at fed state (n = 6)**	**Colonic administration (n = 3)***
Dose (mg/kg)	2	12	12	12
C_0 (ng/mL)	410.8 ± 46.4	-	-	-
C_{max} (ng/mL)	-	149.9 ± 34.8	346.6 ± 50.3**	302.2 ± 37.1
T_{max} (h)	-	4	7	1.5
CL (L/h/kg)	2.9 ± 0.4	-	-	-
V_{ss} (L/kg)	9.9 ± 1.1	-	-	-
$AUC_{0\rightarrow t}$ (h·ng/mL)	673.1 ± 98.1	960.9 ± 231.2	2570.2 ± 382.4**	978.9 ± 143.8
Bioavailability (%)	-	23.8 ± 5.7	63.6 ± 9.5**	24.2 ± 3.6

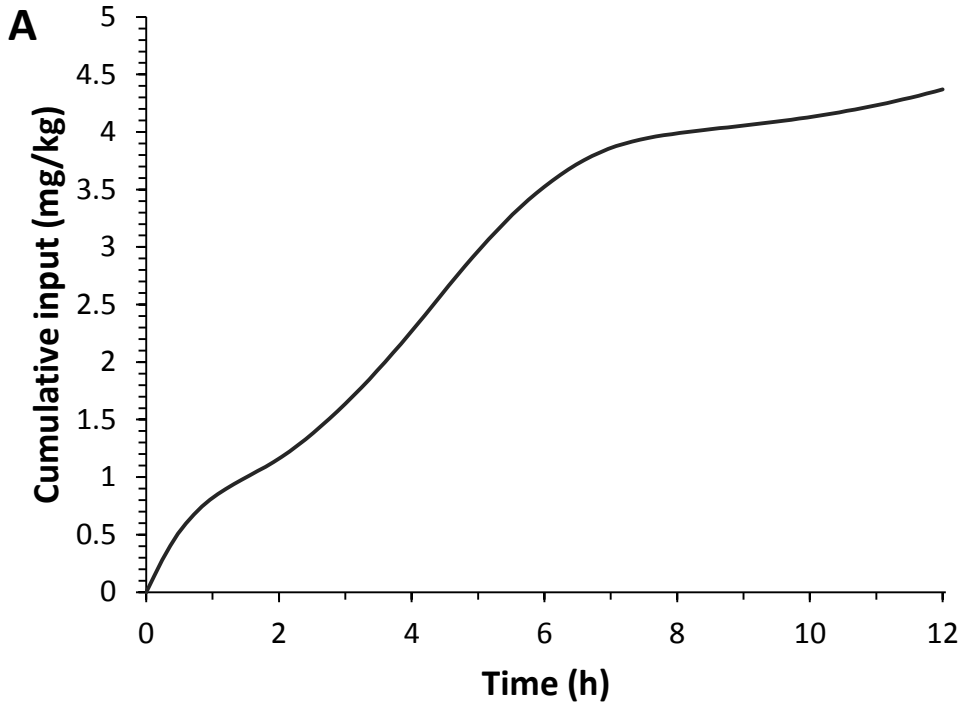
* Colonic administration was performed by infusion for 1 h through a cannula inserted to the cecum.

** Significantly different compared to oral administration at fasted state (p <0.05).

C_0, concentration extrapolated to time zero; C_{max}, maximum concentration observed; T_{max}, time of maximum concentration observed; $t_{1/2}$, elimination half-life; CL, clearance; V_{ss}, volume of distribution at steady state; $AUC_{0\rightarrow t}$, area under the curve from time zero to the last observed point.

3.2. Deconvolution of plasma concentration-time profiles

Deconvolution of the plasma concentration-time profiles obtained from *in vivo* oral bioavailability evaluation was performed in order to understand the absorption or input function of DF030263 following oral administration. The results of cumulative input or input rate vs time are shown in Figure 3. It can be seen from Figure 3A that the absorption shows a biphasic input function; the first phase between 0-1 h and the second phase between 2-6 h. This becomes more apparent in Figure 3B where two peaks are shown from the input rate vs time graph. These results indicated possibility of existence of two absorption windows for DF030263 along the gastrointestinal tract.



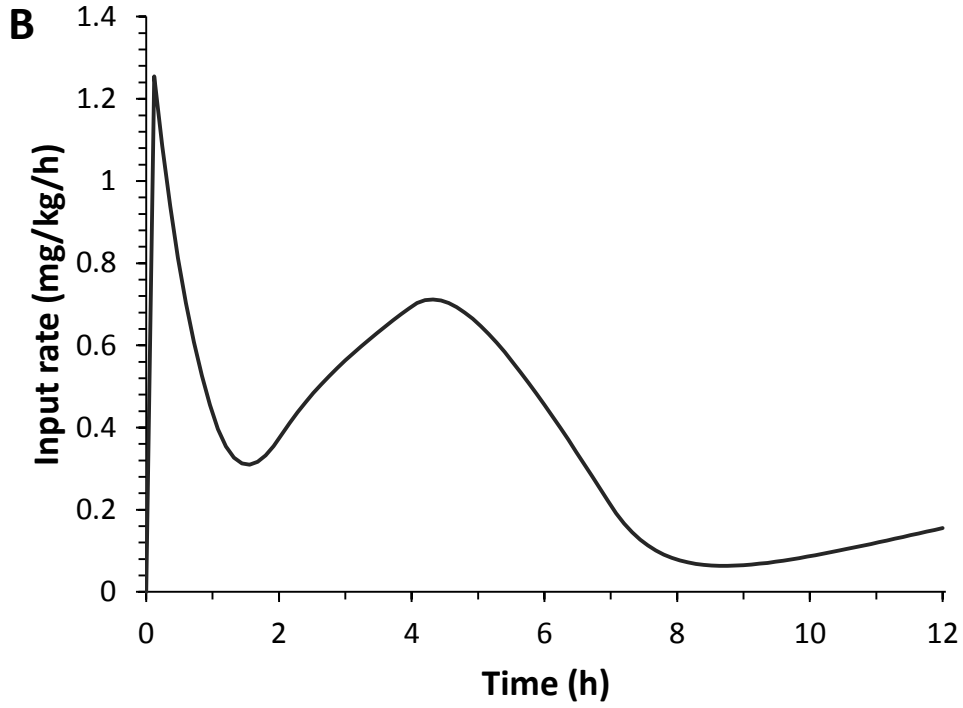


Figure 3. Deconvolution results of plasma concentration-time profiles obtained from oral bioavailability evaluation of DF030263. **A**, cumulative input vs time; **B**, input rate vs time.

3.3. *In vitro* solubility tests

In vitro solubility tests were conducted with simulated fluids of FaSSGF, FaSSIF and FeSSIF to provide information on solubilization behavior of DF030263 along the gastrointestinal tract. The pH values of these simulated fluids are commonly used to represent the mean pH found in the gastrointestinal tract. However, different pH values are observed in different segments of the gastrointestinal tract *in vivo* [16-18]. Therefore, in this study, the pH of each simulated fluid was adjusted to represent the pH range found *in vivo* and solubility of DF030263 was tested at each pH level. In general, DF030263 showed higher solubility in acidic environment (Figure 4). DF030263 exhibited pH-dependent solubility especially in FaSSIF where the solubility decreased steeply when the pH increased above 6.6. This indicated that there could be high probability of precipitation in segments of gastrointestinal tract with higher pH levels. This pH-dependent decrease was less apparent in FeSSIF. Solubility in water was 88.3 ± 2.6 μM.

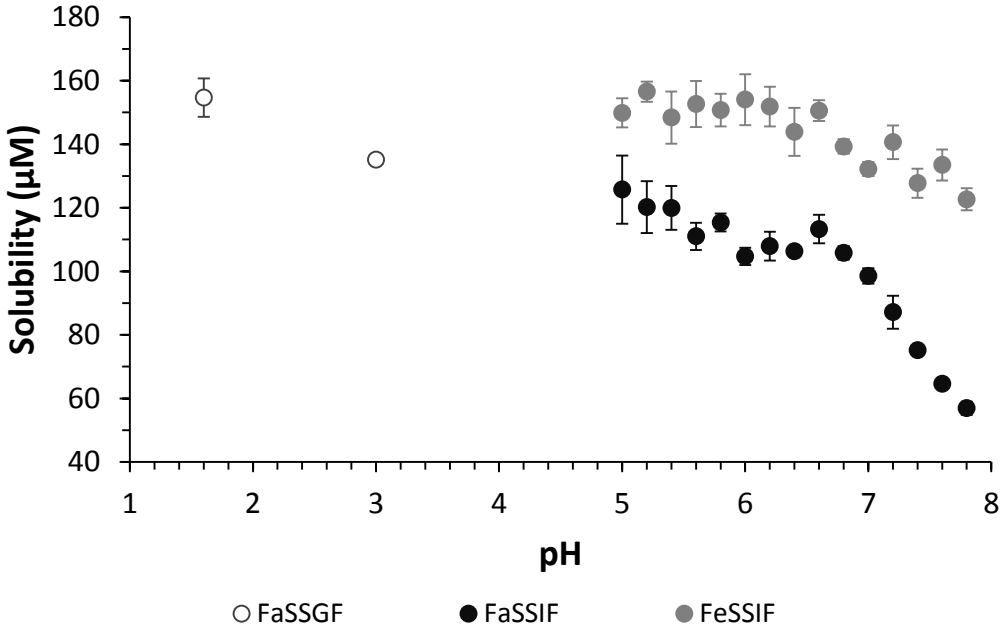


Figure 4. Solubility of DF030263 in FaSSGF, FaSSIF and FeSSIF at various pH (n = 3). FaSSGF, fasted state simulated gastric fluid; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid.

3.4. *In silico* simulation of intestinal absorption

In silico simulation was performed to predict intestinal absorption of DF030263 *in vivo*. GastroPlus™ utilizes advanced compartmental absorption and transit (ACAT) model for their intestinal absorption simulation [19], which compartmentalizes the gastrointestinal tract into nine different compartments. Therefore it was able to predict the fraction of the dose that will be absorbed at the different compartments. The simulation results at both fasted and fed states of the rat are shown in Figure 5. The results show that DF030263 is predicted to be firstly absorbed at the proximal regions of the small intestine and then has an additional absorption window in the cecum and colon.

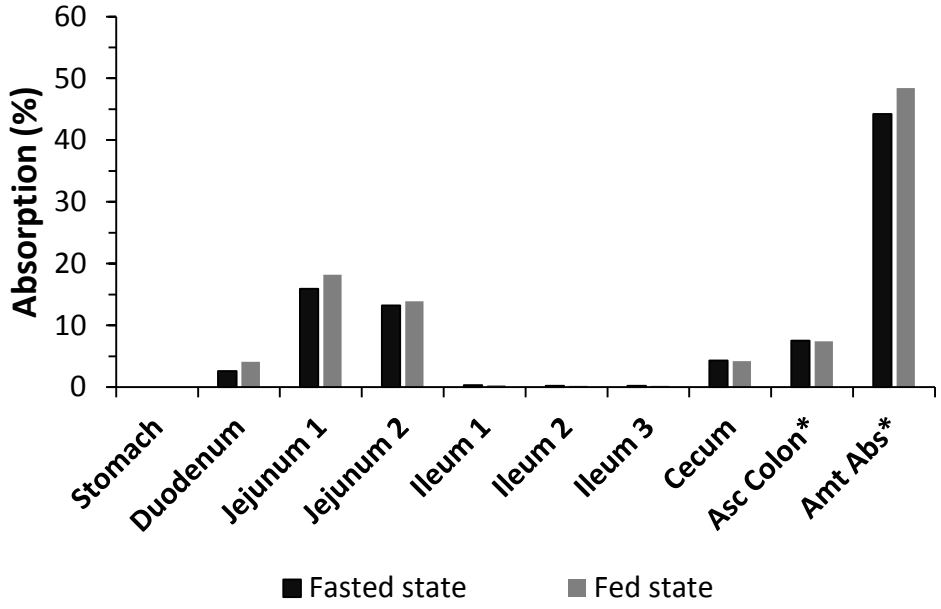


Figure 5. *In silico* simulation of intestinal absorption of DF030263 at each compartment of the gastrointestinal tract. *Asc Colon, ascending colon; Amt Abs, total amount absorbed in the gastrointestinal tract.

3.5. Colonic and postprandial administration of DF030263

Following the above results, colonic and postprandial administration of DF030263 were evaluated in order to improve oral bioavailability of DF030263. Colonic administration was

delivered by infusion through the cannula inserted to the cecum for 1 h to mimic controlled release of the drug in the large intestine. Postprandial oral gavage administration was performed on rats that had free access to food and water throughout the experiment. The double-peak phenomenon observed after administration by oral gavage at fasted state was not seen in the case of postprandial administration or colonic delivery (Figure 6). Colonic administration resulted in rapid absorption (T_{max} = 1.5 h) but no improvement in bioavailability was noted (Table 3). On the other hand, oral administration at fed state resulted in significantly higher C_{max} and bioavailability compared to fasted state.

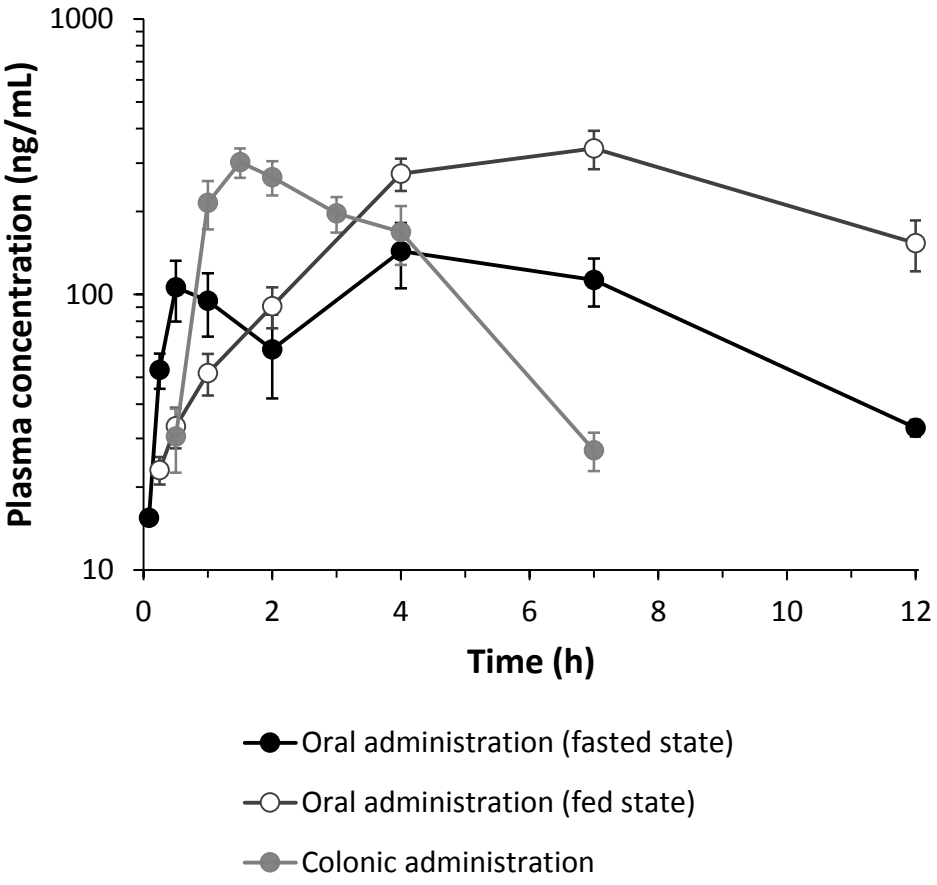


Figure 6. Mean plasma concentration-time profiles of DF030263 in rats following oral administration at fed state (12 mg/kg, n = 6) and colonic administration (12 mg/kg, n = 3).

4. Discussion

4.1. *In vivo* oral bioavailability evaluation and the double-peak phenomenon

In the initial *in vivo* oral bioavailability evaluation, DF030263 showed mean oral bioavailability of 23.8%. Interestingly, a distinct double-peak phenomenon was observed and it only appeared after oral administration of DF030263 (Figure 2). Similar double-peak phenomenon following oral administration has been reported for a number of drugs including acetaminophen [20], alprazolam [21], cimetidine [22], epinastine [23], furosemide [24], pafenolol [25], ranitidine [26] and veralipride [27]. This phenomenon is usually attributed to the following three main causes [21, 26, 28-31]: 1) enterohepatic recirculation where the drug in the systemic circulation is secreted via bile and reabsorbed from the gastrointestinal tract; 2) variable absorption properties along the gastrointestinal tract (also called "absorption windows"); 3) gastric emptying time is varied depending on motility of the gastrointestinal tract, gastric pH or the lipidic formulation effect. In all cases, this erratic pattern of absorption can potentially be problematic for CDK9 inhibitors as relatively narrow therapeutic window is known to be one of their potential drawbacks [32].

In the case of DF030263 compound, enterohepatic recirculation was excluded from the possible reasons because the plasma concentration-time profile following intravenous administration did not show a double peak. Variance in gastric emptying time was also unlikely to be the reason because the time between the two peaks was as long as 3-6.5 h. Moreover, all four rats orally administered at fasted state displayed double-peaks. Therefore, absorption window of DF030263 in the gastrointestinal tract was thought to be discontinuous and further studies were conducted to elaborate the two-site absorption windows hypothesis.

4.2. Two-site absorption windows hypothesis

Deconvolution of the plasma concentration-time profiles were conducted to elucidate absorption rate over time for DF030263 following oral administration. Deconvolution represents a mathematical process that can inversely uncover the input function when the oral administration profile is known and drug disposition characteristics are defined by the intravenous administration profile [33]. This allows determination of the absorption characteristics which can often be challenging to measure or quantify *in situ* [34]. As shown in Figure 3, the absorption function of DF030263 exhibited a biphasic process which leads to the two-site absorption.

The two-site absorption windows hypothesis was further supported with the results of the *in silico* simulation using GastroPlus™. The built-in ACAT and generic physiologically-based pharmacokinetic models in the software provide effective predictions of pharmacokinetic profiles in preclinical species and humans [35-38]. In this study, intestinal absorption of DF030263 in different compartments of the rat gastrointestinal tract was predicted. The simulation results predicted clear discontinuation of absorption in the distal region of the small intestine and a second absorption window in the large intestine (Figure 5). Additionally, the secondary absorption phase following oral administration also corresponded to the oral-to-cecal transit time in rats (2-3 h) [25, 39]. This corroborated the assumption that a second absorption window exists in the large intestine. Such site-specific absorption of drugs is known to occur due to properties related to solubility, stability or interaction with luminal contents [40].

The luminal pH levels can differ in different segments of the gastrointestinal tract and therefore *in vitro* solubility tests were carried out in a range of pH using simulated biorelevant media of

FaSSGF, FaSSIF and FeSSIF. The solubility of DF030263 was maintained at the highest levels in the acidic environment of FaSSGF and the lower pH ranges of FaSSIF and FeSSIF (Figure 4). This is explained by the fact that DF030263 is a weak base with a pK_a of 10.7 (predicted by ACD/Labs, Toronto, Canada) and therefore would be solubilized more efficiently in such acidic environment. When the pH level was increased above 6.6, DF030263 showed pH-dependent solubility with decreasing solubility especially in the FaSSIF. This suggested that DF030263 could be precipitating in the regions of the gastrointestinal tract where the pH is relatively high, such as the distal small intestine. The luminal pH in the rat small intestine increases as it reaches the distal region; from pH 6.5 in the duodenum to 7.1 in the ileum [16]. This possibility of precipitation in the small intestine with higher pH has been acknowledged especially for weakly basic compounds and it has been put forward as a critical drug development obstacle, significantly limiting the oral bioavailability of these compounds [41]. The luminal pH drops again when it reaches the colon to 6.6 [16], which can provide opportunity for DF030263 to resolubilize and be available for absorption again. The pH-dependent solubility was therefore thought to be the main reason behind the two-site absorption windows. Since humans have a similar luminal pH levels pattern to rats, this phenomenon is likely to occur in humans as well [18].

4.3. Exploitation of the two-site absorption windows

In order to improve the oral bioavailability of DF030263, the pH-dependent solubility and potential precipitation in the distal region of the small intestine had to be mitigated. Colonic administration mimicking controlled release of DF030263 in the large intestine and postprandial oral administration were tested for this purpose. Deconvolution and *in silico* simulation of the intestinal absorption both indicated a second absorption window in the large intestine. Additionally, when partial area under the curve (AUC) is calculated for the oral

administration profile at fasted state, the window between 2-12 h accounts for 86% of the total AUC. With all the above-mentioned factors, colonic administration was thought to possibly improve the bioavailability of DF030263.

Colonic administration was performed via a cannula inserted into the cecum and DF030263 solubilized in the dosing vehicle was infused for 1 h. Therefore it would not pass through the distal region of the small intestine where the pH is relatively higher and the solubility of DF030263 is lower. Consequently, potential loss in absorption of DF030263 due to the precipitation-resolubilization process can be avoided.

As shown in Figure 6, colonic administration resulted in rapid absorption and only single peaks were observed in all rats tested. This is consistent with a previous study where pafenolol, which had double-peak following oral administration, showed a single peak when administered through an intraintestinal cannula to target a specific absorption window in the gastrointestinal tract [25]. In spite of the single peak, colonic delivery did not improve the bioavailability of DF030263, but it was rather comparable to the oral administration at fasted state (Table 3). As a result, it was confirmed that the large intestine is indeed an absorption window but oral bioavailability cannot be improved by controlled release to this site. It is likely that the amount of dose that can be absorbed from the large intestine has been already absorbed from simple oral administration.

Postprandial oral administration was also tested mainly based on the premise that solubility is enhanced in fed conditions. The *in vitro* solubility results in Figure 4 clearly show that in FeSSIF, the solubility was higher and was less dependent on the change of pH. The presence of food in the gastrointestinal tract modifies the luminal contents which leads to changes in pH,

buffer capacity and surface tension thereby affecting solubilization of drugs [41]. Therefore less precipitation of drugs can be anticipated at fed state especially for weak bases with poor solubility [41] which means that more drug can be available for absorption. Also an important food-effect is the delay in gastric emptying which alters the gastrointestinal transit time [40]. This delay can allow the drug to reside longer time at the first absorption window which is the proximal region of the small intestine (Figure 5).

The plasma concentration-time profile following postprandial oral administration showed a single-peak with substantially improved bioavailability (Figure 6 and Table 3). Avoidance of the double-peak phenomenon by the food-effect has been previously reported [31, 39] but did not necessarily relate to increase in bioavailability. In the case of DF030263, higher solubility, less precipitation and prolonged exposure to the first absorption window had significantly positive effects towards improving the oral bioavailability. Delayed gastric emptying had also caused extended absorption phase resulting in T_{max} of 7 h. It is also noteworthy that the variability in the bioavailability was reduced at fed state (Table 3), which is crucial for drugs such as CDK inhibitors where the narrow therapeutic window is a limitation [32].

To note, there could be interspecies differences between the rats and the humans in the gastrointestinal physiology and luminal contents which might result in different food effects. However, the changes of pH between fasted and fed states are similar between the two species and the rat intestinal fluid has slightly higher concentration of bile salt and phospholipid compared to the FeSSIF [17, 42, 43]. Therefore, similar pattern of food effect could be expected in humans although the extent might vary.

5. Conclusion

In conclusion, this study demonstrates an *in vitro-in vivo-in silico* approach in improving the oral bioavailability of DF030263, a promising candidate for treatment of CLL. The two-site absorption windows hypothesis was suggested and supported by *in vitro* and *in silico* studies following observation of a double-peak phenomenon *in vivo*. Exploitation of the two-site absorption was attempted in order to improve the bioavailability. Colonic administration confirmed that the large intestine is a second absorption window but indicated that controlled release to the colon would not enhance the drug exposure. Instead, oral administration at fed state took advantage of the food-effect in terms of improved solubilization, reduced precipitation and delayed gastrointestinal transit time, thereby increasing the bioavailability.

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