Drug Interaction Studies Reveal That Simotinib Upregulates Intestinal Absorption by Increasing the Paracellular Permeability of Intestinal Epithelial Cells

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Summary: Hypothesis: Simotinib hydrochloride (SIM6802), which is a new epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), is often prescribed for cancer patients with comorbidities and has serious adverse effects on gastrointestinal physiology. The drug-drug interactions (DDIs) between simotinib and other drugs in combination and the underlying mechanism of its gastrointestinal toxicity remain unclear. We hypothesized that the DDIs and the gastrointestinal toxicity of simotinib were related to its effects on the permeability of the intestine. Methods: To determine the intestinal absorption capacity, pharmacokinetic studies and an in situ loop assay were used. The intestinal permeability was measured by a Caco-2 Transwell model. Real time PCR and Western blots were applied to detecting the expression changes of cell junction genes. Results: Our research demonstrated that simotinib upregulated the absorption of cefaclor, valaciclovir and acyclovir. The increase of non-selective absorption was caused by the low expression of cell junction gene afadin-6 and the increase in paracellular permeability in intestinal epithelial cells after simotinib treatment. Conclusion: These findings revealed that simotinib upregulated intestinal absorption by increasing the paracellular permeability of intestinal epithelial cells. Our research provides theoretical bases for better formulation of EGFR-TKIs to alleviate adverse gastrointestinal effects and also provides guidance for clinical administration of simotinib.

Keywords: absorption; adverse drug reactions; drug-drug interactions; membrane permeability; tight junction; pharmacokinetics

Introduction

The receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), have been major targets for anticancer therapy because they are abundantly expressed on the cell surface and play an important role in the modulation of growth factor signaling in cancer cells. EGFR-tyrosine kinase inhibitor (EGFR-TKI), which blocks the molecular signaling pathways induced by EGFR, is a class of targeted chemotherapy medications widely used today.

Simotinib hydrochloride (SIM6802) is a new EGFR-TKI anti-tumor drug developed by Jiangsu Simcere Pharmaceutical Research Company in Nanjing (Fig. 1). Like many other EGFR-TKIs, it is prescribed for prolonged periods, often in patients with comorbidities. Therefore, the risk of DDIs has to be taken into consideration. But few studies have been carried out to investigate the DDIs with simotinib to date. Because infection is a common problem in persons with cancer, we focused on the DDIs between simotinib and co-administered anti-infective drugs such as cefaclor, valaciclovir and acyclovir. Oral administration is the major delivery route for simotinib, and high dosage of EGFR-TKI administration results in some gastrointestinal side effects, such as nausea, vomiting, diarrhea and emaciation. Simotinib also displayed the same adverse gastrointestinal effects in our preliminary studies. The maximum tolerated dose (MTD) of simotinib is 500 mg. Over this dosage range, patients would suffer from nausea, vomiting, diarrhea and emaciation. To reveal the drug interactions between simotinib and widely prescribed co-medications and to understand the mechanism of the adverse effect, we focused on simotinib and some commonly prescribed anti-infective drugs.
gastrointestinal effects, we investigated the effect of simotinib on the drug exposure of other drugs in combination and its effect on intestinal absorption.

The first barrier for the absorption of nutrients and electrolytes is the intestinal mucosal epithelium. Dysfunction of the intestinal mucosal barrier under certain physiological conditions will allow various small molecules to permeate across intestinal epithelial cells mainly through paracellular pathways by passive processes. Three cell junctions can be identified in intestinal epithelia: desmosomes, adherens junctions (AJs), and tight junctions (TJs). The AJs, which are formed by cadherin-catenin interactions, are important in the mechanical linkage of adjacent cells. On the other hand, the TJs, which consist of 4 transmembrane proteins—occludin, claudins, junctional adhesion molecules (JAMs) and tricellulin—are the apical-most junctional complex and responsible for sealing the intercellular space. Afadin-6, which is a target of the EGFR/Ras/MAPK signaling pathway interacts with ZO-1 and serves as a peripheral component of TJs in epithelial cells. Several articles have reported that EGF and EGFR play a crucial role in the protection of intestinal epithelial permeability. In 2004, Sheth et al. reported that EGF prevented acetaldehyde-induced paracellular permeability in Caco-2 cells. In 2007, Singh et al. found activation of EGFR regulated claudin expression and increases in transepithelial electrical resistance (TEER). Because simotinib is an EGFR-TKI that blocks the activation of EGFR, we speculated that the adverse gastrointestinal effects might be caused by increased paracellular permeability of intestinal epithelial cells.

Our results showed that simotinib increased the exposure of other drugs in combination, such as cefaclor, valaciclovir and acyclovir. A Caco-2 cell model revealed that simotinib increased the paracellular permeability of intestinal epithelial cells, and that increasing paracellular permeability was partially regulated by decreasing afadin-6 levels.

Materials and Methods

Materials: Simotinib was provided by Jiangsu Simcere Pharmaceutical Research Company. Cefaclor was purchased from Shanghai JianKun Pharmaceutical Co., Ltd. (Shanghai, China). Valaciclovir and acyclovir were purchased from Kunming YuanRui Pharmaceutical Co., Ltd. (Kunming, China). The Caco-2 cells were obtained from XiangYa Central Experiment Laboratory in Central South University (Changsha, China). Cell culture reagents were purchased from Hyclone (Thermo Fisher Scientific, Beijing, China).

Animals: Male and female SD rats (body weight 200 ± 20 g) were supplied by the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were acclimatized to the experimental unit for a week before the experiments. The rats were housed under automatically controlled light cycles and temperature. Ranges of temperature and humidity measured during the study were 23 ± 1°C and 55 ± 5%, respectively. The studies were approved by the Animal Ethics Committee of The Third Xiangya Hospital of Central South University. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Pharmacokinetic studies: The rats were administrated normal saline or drugs suspended in normal saline for 5 days. Blood was collected before or 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, or 6 h after last administration. The plasma was immediately separated by centrifugation at 5,000 rpm for 10 min at 4°C. All samples were stored at −20°C until being analyzed. The plasma concentrations of drugs were detected by high-performance liquid chromatography with UV detection (Agilent 1200, Agilent Technology Inc., Palo Alto, CA). For cefaclor detection, a C18 column 4.6 × 150 mm (Agilent) was used. The mobile phase consisted of 20% methanol and 80% water with 3% acetic acid, and was delivered at a flow rate of 1 ml/min. For valaciclovir and acyclovir detection, a C18 column 4.6 × 250 mm (Agilent) was used. The mobile phase consisted of 93% methanol and 7% water with 0.2% formic acid, and was delivered at a flow rate of 1 ml/min. The pharmacokinetic parameters and statistical analysis were calculated by Phoenix WinNonlin 6.0 (Pharsight Corporation, St. Louis, MO).

In situ loop assay: Rats were anesthetized with chloral hydrate (China National Pharmaceutical Group, Shanghai, China) and a vertical midline incision was made on their abdomens. A jejunal or ileum loop (30 cm in length) was prepared by closing both ends with sutures, then perfused with Hank’s balanced salt solution using a peristaltic pump (Letter of Shanghai ZhiSun Instrument Co., Ltd., Shanghai, China) until the outflow became visually clear. Fluorescein sodium (Solarbio, Beijing, China) was dissolved in 20 ml Hank’s balanced salt solution to a final concentration of 5 µg/ml, and then injected into the jejunal or colonic loop. Samples of 100 µl perfusate were collected from the tubes connected with the perfused intestine at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h. Fluorescence was measured by spectrofluorophotometer (RF-5301PC, Shimadzu, Kyoto, Japan) with excitation at 490 nm and emission at 515 nm. Final results were normalized to the internal surface area of the tested intestines.

Cell culture: Caco-2 cells were maintained in high glucose DMEM supplemented with 10% FBS, 1% NEAA, 1% l-glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) at 37°C in a humidified incubator (Thermo, Marietta, GA) with 5% CO2. The culture medium was changed 24 h after seeding and every other day.

MTT cell viability assay: Cells were seeded on 96-well plates at a density of 2 × 104 cells/well, and treated with different concentrations of simotinib for 5 days. Then sample wells were treated with 20 µl 5 mg/ml MTT solution (Sigma, St. Louis, MO) and incubated at 37°C for 4 h. After removal of the culture solution, the formazan products in sample wells were resolved with 150 µl DMSO by shaking for 10 min. The absorbance of formazan was measured at 490 nm on a microplate reader (Biotek, Winooski, VT). Cell viability was calculated as below:

Cell viability (%) = mean OD value of experimental group/mean OD value of negative control group × 100%

Cell permeability assay: The Caco-2 cells were seeded onto polyester membranes in 12-well Transwell® plates (Corning Incorporated, Corning, NY) at a density of 2 × 105 cells/well, and grown for 21 days. To monitor the cell monolayer integrity, the TEER was detected with the EVOM2 epithelial cell voltage resistance meter (WPI, Sarasota, FL); and the alkaline phosphatase

Fig. 1. The chemical structure of simotinib
Simotinib Increases Intestinal Membrane Permeability

Simotinib was administered into rats to investigate its effects on intestinal membrane permeability. The experimental procedures included real-time PCR, Western blotting analysis, and pharmacokinetic analysis.

**Real-time PCR**: Total RNA was isolated from rat tissues using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified using a spectrophotometer. Reverse transcription of RNA to cDNA was performed using the M-MLV First Strand Kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed using the resulting cDNA with the All-In-One qPCR Mix (GeneCopoeia, Rockville, MD) on a fluorophore-emitting spectrophotometer (RF-5301PC, Shimadzu) with excitation at 490 nm and emission at 515 nm.

**Western blotting analysis**: Cell or tissue lysates were separated by polyacrylamide gel electrophoresis and transferred to PVDF membranes. Proteins were detected using antibodies against specific proteins (tubulin, afadin-6, and beta-tubulin, Sigma). The blots were developed using a chemiluminescence detection system (Bio-Rad, Hercules, CA).

**Statistical analysis**: Data were expressed as mean ± SD. The statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. A probability level of p < 0.05 was considered statistically significant.

**Results**: Simotinib increased the absorption of cefaclor, valaciclovir, and acyclovir. This effect was accompanied by a significant decrease in body weight and food intake in rats treated with simotinib. The results suggest that simotinib may have adverse gastrointestinal effects in whole animals.

**Fig. 2**: Simotinib reduced the rat body weights and food intake

(A) Rat body weights were decreased during 5 days administration of simotinib. (B) Food consumption of rats was reduced during 5 days administration of simotinib. Error bars represent standard error of mean (n = 6).
of other drugs in combination, we checked the plasma concentration of cefaclor, which is a second-generation cephalosporin antibiotic used to treat secondary infections resulting from cancer and cancer treatments. Twelve rats were divided into two groups to receive either simotinib (92.7 mg/kg/d suspended in normal saline 

via intragastric gavage) or normal saline administration for 7 days. A toxic high dose of simotinib was used in the study to test the adverse effects on intestinal epithelia. In the last administration, both experimental and control groups received an intragastric gavage of 51.4 mg/kg cefaclor. Blood was collected before or 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, or 6 h after administration, and the plasma concentration of cefaclor was detected by HPLC. We found simotinib increased the exposure of cefaclor. The value of AUC, Cmax, Tmax and T1/2 were all significantly higher in the simotinib-administered group than in the control group, which indicated the increase of absorption and decrease of elimination (Fig. 3A and Table 1).

Because cefaclor is a substrate of the intestinal transporter Pept1, which recognizes the peptide bond,11) we hypothesized that simotinib increased the exposure of cefaclor by upregulating the function of Pept1. Therefore, we assessed two other drugs: acyclovir and valaciclovir. Acyclovir, which does not contain a peptide bond in its structure, does not interact with Pept1. But valacyclovir, which is the l-valyl ester form of acyclovir, is a substrate of Pept1, and the prodrug valacyclovir is nearly completely converted to acyclovir and l-valine by first passage through the intestine and liver.12) Twenty-four adult rats were randomly allocated to four groups of 6 rats each: the acyclovir experiment group (41.4 mg/kg/d simotinib for 7 days plus 308.5 mg/kg acyclovir at the last administration), the acyclovir control group (saline for 7 days plus 308.5 mg/kg acyclovir at the last administration), the valacyclovir experiment group (41.4 mg/kg/d simotinib for 7 days plus 46.3 mg/kg valacyclovir at the last administration), and the valacyclovir control group (saline for 7 days plus 46.3 mg/kg valacyclovir at the last administration). To guide the clinical application, the dosage of simotinib was changed from the toxic high dose to the common clinical dose. Pharmacokinetic data analysis showed simotinib increased exposure of acyclovir in both the acyclovir and valacyclovir experiment groups. The AUC of acyclovir in the valacyclovir experiment group was significantly higher than in the valacyclovir control group. The AUC and Cmax of the acyclovir experiment group were significantly higher than those of the acyclovir control group (Fig. 3B and Table 1).

These data demonstrated that simotinib did not increase the exposure of other drugs in combination by enhancing the activity of Pept1 but by increasing the permeability of intestinal epithelial cells, although without excluding the effects of hepatic microsomal enzyme or renal transporters.

**In situ loop assay showed simotinib increased the intestinal absorption:** The oral administration model is too complicated to reveal the underlying mechanism for intestinal absorption of drugs because it cannot rule out the effects of hepatic microsomal enzyme or renal transporters. To investigate direct effects of simotinib on the intestinal absorption of drugs, we performed an in situ loop assay. Twelve rats were divided into two groups to

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<th>Tmax (h)</th>
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<th>Cl/F (L/h/kg)</th>
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Pharmacokinetic studies showed that simotinib increased the Cmax or AUClast of cefaclor, valaciclovir and acyclovir.

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receive either simotinib (41.4 mg/kg/d suspended in normal saline via intragastric gavage) or normal saline administration for 7 days. Fluorescein sodium was used as a probe. The absorption rate of fluorescein sodium was increased after simotinib treatment and the cumulative absorptions of fluorescein sodium showed significant difference between those 2 groups after 2 h perfusion (Fig. 4). These data gave us direct evidence of the intestinal absorption-enhancing effects of simotinib.

**Simotinib increased paracellular permeability in a Caco-2 cell model:** To test the effects of simotinib on cellular permeability, we generated a Caco-2 cell Transwell model. First, we tested the cytotoxicity of simotinib on Caco-2 cells. Caco-2 cells were treated with different concentrations of simotinib for 5 days, and the cell viability was measured by MTT staining. The results showed that Caco-2 cells could survive under the treatment with less than 0.1 µM (including 0.1 µM) simotinib (Fig. 5A). Then, the Caco-2 cells were grown in Transwells for 21 days until the TEER and ALP activity reached a plateau (Supplementary Fig. S1 and Table S2). To evaluate the permeability of Caco-2, fluorescein was used as a paracellular transport marker and propranolol was used as a passive transcellular transport marker. Different concentrations of simotinib treatment for 5 days resulted in a concentration-dependent increase in fluorescein permeability (Figs. 5B and 5C). The unidirectional (AP to BL) apparent permeability coefficient (P_app) was increased from 65.5 ± 5.17 × 10⁻⁸ cm/s in the non-treatment group to 81.3 ± 2.57 × 10⁻⁸ cm/s in the 0.1 µM simotinib treatment group (n = 3, p < 0.05), and 87.0 ± 6.63 × 10⁻⁸ cm/s in the 0.5 µM simotinib treatment group (n = 3, p < 0.01). But there was no significant difference in propranolol permeability in any experimental groups (Fig. 5D). The TEER was also unchanged after simotinib treatment (Supplementary Fig. S2). These data indicate that simotinib upregulates the intestinal absorption by increasing the paracellular but not the passive transcellular permeability of intestinal epithelial cells.

**Fig. 4. Simotinib increased the intestinal absorption in an in situ loop assay**
(A) The cumulative absorptions of fluorescein sodium per square centimeter internal surface of intestine were plotted with the increase of time in both simotinib- and saline-administrated groups. (B) The absorption rates of fluorescein sodium were calculated for both simotinib- and saline-administrated groups. Error bars represent standard deviation (n = 6).

**Fig. 5. Simotinib changed the permeability of a Caco-2 cell monolayer**
(A) Survival rates of Caco-2 cells after treatment with different concentrations of simotinib were measured by MTT staining. Error bars represent standard deviation (n = 6). (B) The flux rates of fluorescein after treatment with different concentrations of simotinib. (C) The P_app (AP to BL) of fluorescein was increased after simotinib treatment in a concentration-dependent manner. (D) The P_app (AP to BL) of propranolol was not changed after simotinib treatment. Error bars represent standard deviation (n = 3).

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Simotinib reduced the expression of afadin-6: Paracellular permeability was regulated by cell junction genes. Reducing expression of cell junction genes led to increased paracellular permeability. A previous study reported that several cell junction genes were modulated by EGF, so we tested whether simotinib increased the paracellular permeability by modulating the expression of those cell junction genes. RNA and protein were extracted from Caco-2 cells treated with different concentrations of simotinib (0, 0.005, 0.02, 0.1, 0.5, 2.0 µM). Real time RT-PCR data showed that only afadin-6 mRNA levels were reduced by simotinib in a concentration-dependent manner. Other cell junction genes, such as occludin, β-catenin, tricellulin, CTNNA and E-cadherin, were unchanged after simotinib treatment. The mRNA levels of ZO-1 and CLDN1 increased slightly after simotinib treatment (Fig. 6A). Western blot results also confirmed that simotinib decreased afadin-6 protein levels at high concentrations (0.1, 0.5 and 2.0 µM), but not at low concentrations (0.005 and 0.02 µM) (Fig. 6B).

To confirm our results in vivo, RNAs and proteins from the internal surface of rat intestines were collected in both simotinib-(41.4 mg/kg/d suspended in normal saline via intragastric gavage for 7 days) and normal saline-administrated rats. Real time PCR data demonstrated that the mRNA levels of afadin-6 were slightly decreased in simotinib-treated rats compared to the saline-administrated rats; and the other genes, such as ZO-1, occludin, tricellulin and CLDN1, remained the same (Fig. 6C). Western blot results also showed that afadin-6 protein levels were slightly decreased in simotinib-treated rats (Fig. 6D).

These data demonstrate that simotinib increases paracellular permeability by reducing the expression of afadin-6.

Discussion

Although targeted drugs are generally less toxic than traditional chemotherapy agents, EGFR-TKIs still result in some adverse effects that need to be managed in patients. Because EGFR is highly expressed on epithelial cells, such as those lining the skin and the gastrointestinal tract, rash and gastrointestinal side effects are the most common side effects of EGFR-TKI noted in clinical trials. In phase III clinical trials, the incidence of diarrhea with 150 mg erlotinib varied from 40% to 67.9% in both first-line combination therapy and second-line monotherapy, and 6–12.4%
of patients experienced severe reactions (grade 3 or higher).\textsuperscript{16–18} Gefitinib 250 mg resulted in gastrointestinal side effects in 27–58.2% patients in both first-line and second-line therapy, and 2.5–9.9% of patients experienced severe reactions.\textsuperscript{19–22} Afinittin 50 mg caused diarrhea in 87% patients after failure of chemotherapy and erlotinib or gefitinib; the incidence of severe reactions was 17%.\textsuperscript{23} Nowadays, dietary changes or antidiarrheal medications are the general medical management for EGFR-TKI-caused gastrointestinal side effects,\textsuperscript{24} but the underlying molecular mechanism of gastrointestinal tract-specific toxicity induced by EGFR-TKI has remained unclear. Our results demonstrated that simotinib reduced the expression of the cell junction gene afadin-6 and increased the paracellular permeability of intestinal epithelial cells, which led to the increase of non-selective absorption and loss of nutrients and electrolytes. Cell junction integrity is important for intestinal absorption; ZO-1, occludin, tricellulin and CLDN1 are key components of TJ seals.\textsuperscript{25} Afinittin-6 is not a key component of tight junctions, but it interacts with ZO-1 through the two proline-rich regions of afadin and the SH3 domain of ZO-1,\textsuperscript{26} and serves as a peripheral component of TJs in epithelial cells.\textsuperscript{5} It helps the nectin to recruit ZO-1 to cell-cell adhesion sites.\textsuperscript{27} We did not observe any expression changes of TJ genes in either \textit{in vivo} or \textit{in vitro} studies. But we did observe the expression decrease of afadin-6 in both \textit{in vivo} and \textit{in vitro} studies. We think simotinib down-regulates the expression of afadin-6, so the recruitment of ZO-1 to cell-cell adhesion is impeded and the tight junction formation is blocked. Although the expression level of ZO-1 is not affected by simotinib, its localization is changed.

To attenuate the adverse gastrointestinal effects of EGFR-TKI, some groups tried to use adjuvants, such as glucagon-like peptide-2\textsuperscript{28} and curcumin,\textsuperscript{29} to recover the gastrointestinal function. We believe that the adverse gastrointestinal effects mainly occurred by the direct interaction between the EGFR-TKI and intestinal epithelial cells. A better drug delivery system, such as a liposome-embedded particulate drug delivery system, could alleviate the adverse gastrointestinal effects. Our future direction will focus on particulate drug delivery systems for EGFR-TKI administration.

Simotinib increases the exposure of other drugs in combination. The AUC in cefaclor, valaciclovir and acyclovir group are all increased. The $C_{\text{max}}$ of cefaclor and acyclovir are both increased compared to their saline controls, which demonstrates the increase of intestinal absorption. \textit{In situ} loop assay also gives us direct evidence of the intestinal absorption-enhancing effects of simotinib. To rule out the effects of hepatic microsomal enzymes, we also tested the effects of simotinib on the activities of CYP450 by rat liver microsome assay. Simotinib showed no significant inhibitory effect on the activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6 or CYP3A4 in the concentration range of 0–200 μM. There was only a little inhibitory effect of simotinib on the activity of CYP2E1 and the IC50 value was 75.26 μM. (For commercial reasons, we cannot show the details.) Therefore, we do not think simotinib increases the drug exposure by inhibiting drug metabolism, but by increasing drug absorption. We know the $C_{\text{max}}$ in the valaciclovir group does not increase very much, because we detect the plasma concentration of acyclovir, which is a converted product of valaciclovir. The hepatic metabolism changes the concentration of the prodrug (valaciclovir). A delayed $T_{\text{max}}$ from 0.61 h to 1.08 h and an increased $T_{1/2}$ from 1.25 h to 2.25 h are detected in the cefaclor experiment group, which suggests that simotinib has an effect on cefaclor elimination. Since cefaclor is not metabolized and excreted \textit{via} urine, simotinib should inhibit one or more renal transporters, such as Oat1, Pep1, and Pep2.\textsuperscript{30} We will focus on the effects of simotinib on the renal transporters in the future. Another contribution of our research is to guide the clinical administration of simotinib. Since simotinib increased the intestinal absorption, we should reduce the dosage of other drugs when they are used in combination with simotinib.

We used different concentrations of simotinib for \textit{in vivo} and \textit{in vitro} studies. The MTD of simotinib for patients is 500 mg. The calculations for determining the starting dose in humans as extrapolated from animals should use the more appropriate normalization of body surface area. The formula is: Rat dose (mg/kg) = 6.17 (conversion factor) × human dose (mg/kg) (60 kg). So, for \textit{in vivo} studies, we administer 92.7 mg/kg in rats, which represents 900 mg in humans, to study the toxic effects of simotinib on the gastrointestinal tract, and 41.4 mg/kg in rats, which represents 400 mg in humans, to mimic the common clinical dose. The concentration of simotinib in the gastrointestinal tract under the common clinical dose (>500 μM) is much higher than in our \textit{in vitro} studies (0–2 μM) because Caco-2 is a colon cancer cell. Its tolerance to simotinib is much lower than that of the normal intestinal tissue and the IC$_{50}$ is about 0.5 μM. That is why we used a high concentration for \textit{in vivo} pharmacokinetic studies, but used a low concentration for \textit{in vitro} studies.

All together, our research provides theoretical bases for better formulation of EGFR-TKI to alleviate adverse gastrointestinal effects and also provides guidance for clinical administration of simotinib.

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References


(2007).


