

Evaluation of Intestinal Permeability of the Antifungal Compound PD76: Comparison of *in silico* Platforms and *in vitro* Assay in Caco-2 Cell Model

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The compound 2-hydrazinyl-4-(4-methoxyphenyl)thiazole (PD76) is a novel thiazolyl hydrazine derivative with proven antifungal activity against different fungal species, mainly *Candida* and *Cryptococcus*. Considering the advantages of oral route for clinical therapy, the aim of this work was to evaluate the potential intestinal permeability of this new antifungal drug. For the quantitation of PD76, a high-performance liquid chromatography method was developed and fully validated. The cytotoxicity of the compound in Caco-2 cells was analyzed and intestinal permeability of PD76 was assessed by means of the comparison of *in vitro* assay in Caco-2 cells and *in silico* platforms ADMETlab and admetSAR. Cell viability above 70% was obtained at all PD76 studied concentrations. Using Caco-2 cell model, the compound showed apparent permeability coefficients (Papp) of 5.25×10^{-6} and 23.28×10^{-6} cm s⁻¹ in apical-basolateral and basolateral-apical directions, respectively. Experiments performed using verapamil as P-gp inhibitor demonstrated that PD76 is slightly susceptible to active efflux. Both *in silico* platforms inferred that PD76 presents permeability in Caco-2 cells, with Log P values of 2.82 (ADMETlab) and 2.10 (admetSAR). The results obtained in permeability studies showed that PD76 presents moderate intestinal permeability and a promising profile for clinical application.

Keywords: permeability, intestinal absorption, Caco-2 cells, HPLC, *in silico* modeling

Introduction

Invasive fungal infections are a serious public health concern worldwide, especially for immunocompromised patients.¹ Besides the high morbidity and mortality rates related to these diseases, fungal resistance and the emergence of highly pathogenic species are aggravating factors for an efficient treatment.² The current arsenal of antifungal drugs is small and presents several limitations, such as high toxicity, low oral bioavailability and narrow antifungal spectrum. Therefore, the development of new antifungal compounds with high efficacy and safety profile has become increasingly relevant.^{3,4}

In this context, a series of thiazolyl derivatives was developed by our research group, and among them, the compound 2-hydrazinyl-4-(4-methoxyphenyl)thiazole, named PD76 (Figure 1) stood out as a potential antifungal

drug.^{5,6} PD76 showed high activity against different fungal species using broth microdilution method. The minimum inhibitory concentration (MIC) for *Candida albicans*, *Cryptococcus neoformans* and *Cryptococcus gattii* was 0.24 µM, demonstrating an antifungal potential similar to or greater than fluconazole, amphotericin B and itraconazole, drugs already well-established in the antifungal therapy.⁶ The promising activity of PD76 against *Candida* and *Cryptococcus* presents high clinical relevance since these species are related to increased morbidity and mortality rates, besides being susceptible to the development of resistance.⁷

In addition to the assessment of the antimicrobial activity, preclinical studies are essential to substantiate

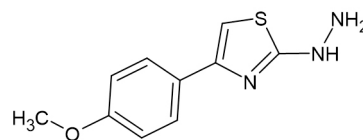


Figure 1. Chemical structure of 2-hydrazinyl-4-(4-methoxyphenyl)thiazole (PD76).

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these compounds as potential drug candidates. Since the oral administration is the preferred route in clinical therapy due to the convenience, cost and safety, the prediction of intestinal absorption and bioavailability is essential during the drug development phase.^{8,9}

Intestinal epithelium is an important barrier that may restrict the oral bioavailability of potential new drugs. Among the available approaches to assess the intestinal absorption during the drug development process, *in vitro* and *in silico* studies emerged as standard tools.¹⁰ Cell monolayer models that mimic the intestinal barrier have been used for *in vitro* permeability studies. Caco-2 cells, a human colon adenocarcinoma, undergo spontaneous enterocytic differentiation when cultured in specific conditions and express the main mechanisms of cellular transport across the intestinal epithelium, as passive drug permeation (both transcellular and paracellular), membrane transporters and active efflux. Therefore, Caco-2 model presents a high correlation with human intestinal mucosa permeation characteristics.^{11,12}

In silico methods to predict membrane permeability involves computational or virtual screening based on the physicochemical characteristics of the compound, such as lipophilicity, H bonding capacity, molecular size, and polar surface area. Quantitative structure property relationship (QSPR) may be employed to assess intestinal absorption with no need for actual compound synthesis. When combined with *in vitro* permeability studies, they reduce the probability of synthesizing poor absorbed compounds, favoring a more effective and less expensive screening.^{10,11}

Thus, the objective of this work was to evaluate the intestinal permeability of the compound PD76, aiming to assess its potential as a novel antifungal drug by oral administration. *In vitro* intestinal permeability was determined by Caco-2 cell model and *in silico* evaluation was performed by computational tools that correlate the chemical structure of the molecule to its intestinal absorption.

Experimental

Chemicals and reagents

PD76 was in-house synthesized following a previously described procedure⁵ using equimolar amounts of thiosemicarbazide and 2-bromo-4'-methoxyacetophenone in methanol, both supplied by Sigma-Aldrich (St. Louis, MO, USA). Product characterization was performed according the procedures described by Franco *et al.*⁶ (purity > 95%). Verapamil hydrochloride was purchased

from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water obtained in a Millipore system (Bedford, MA, USA) was used in the analysis. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Hanks' Balanced Salts Solution (HBSS) were obtained from Gibco® (Grand Island, NY, USA). Acetonitrile (HPLC grade) was from Tedia® (Fairfield, OH, USA), whereas ammonium acetate and formic acid (analytical grade) were from Synth® (Diadema, SP, Brazil). Transwell plates were from Corning® (Cambridge, MA, USA).

Instrumental and chromatographic conditions

The HPLC system (Waters® Alliance, New Castle, DE, USA) consisted of quaternary pump, autosampler, ultraviolet detector (UV), and Empower 3.0 software. Separation was obtained on Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, 5 µm) from Agilent® (Agilent Technologies, Santa Clara, USA), at 25 °C. Different solvents were tested as eluent and the final mobile phase composition was acetonitrile (solvent A) and 2 mM ammonium acetate with 0.1% formic acid (solvent B), at 1.0 mL min⁻¹. Linear gradient elution was used: 30% A at 0 min and 30-88% A from 0 to 6 min. Re-equilibration was performed using 30% A for 2 min. Injection volume was 50 µL and UV detection was performed at 260 nm. UV spectra from 200 to 400 nm were evaluated to determine the wavelength of maximum absorption for PD76 detection.

Preparation of PD76 solutions

Stock solution was prepared by weighting 2.5 mg of PD76 to a 5 mL volumetric flask. Then, 1.25 mL of dimethylsulfoxide (DMSO) and 2.8 mL of ethanol were added and the solution was submitted to ultrasound bath for 5 min. The volume was adjusted with ultrapure water, to obtain a 0.5 mg mL⁻¹ concentration. Working solutions were prepared by diluting the stock solution with HBSS to a final concentration of 10 µg mL⁻¹. All solutions were prepared immediately before the use and filtered through a 0.45 µm membrane.

HPLC method validation

Method validation was performed according to the procedures described by US Food and Drug Administration¹³ and European Medicine Agency.¹⁴

Selectivity of the HPLC method was assessed by the peak purity of PD76 in the sample solutions. In addition, HBSS and all media used in the permeability study were injected in the chromatographic system, to evaluate possible

interfering peaks at the same retention time of PD76. The carry-over effect was evaluated by sequential injections of blank samples before and after the analysis of a PD76 at the upper limit of quantification ($12 \mu\text{g mL}^{-1}$).

Linearity was evaluated by eight-point calibration curves in triplicate, constructed by plotting the peak area *versus* the concentration of PD76. The assayed concentrations were 0.05, 0.1, 0.3, 1.0, 3.0, 6.0, 9.0 and $12 \mu\text{g mL}^{-1}$. Since the calibration standards should be prepared in the same matrix as the samples in the intended study, HBSS media was used to prepare these solutions. The obtained data were subjected to regression analysis using the ordinary least squares method. The curve was accepted if at least 75% of the calibration standards were within $\pm 20\%$ at lower limit of quantification (LLOQ) and $\pm 15\%$ for other levels.

Precision and accuracy of the method were evaluated in the same run (intra run) and in three different runs (inter run). Five concentrations of PD76 (0.05, 0.1, 5.0, 10.0 and $20.0 \mu\text{g mL}^{-1}$) were assayed in quintuplicate in each run. To evaluate the dilution integrity of the procedure, PD76 solution at $20.0 \mu\text{g mL}^{-1}$ was diluted four times fold in HBSS, to obtain $5.0 \mu\text{g mL}^{-1}$. Precision was expressed as relative standard deviation (RSD, in percentage) and accuracy as relative error (RE, in percentage). For the evaluation of the method sensitivity, the LLOQ was set at the lowest concentration that showed accuracy within $\pm 20\%$ and $\text{RSD} \leq 20\%$.

For stability evaluation, PD76 solutions were prepared at 0.1 and $10.0 \mu\text{g mL}^{-1}$ in HBSS, in triplicate. Aliquots of these solutions were analyzed immediately after preparation and the second aliquot was subjected to the conditions of the permeability study (orbital agitation at 50 rpm, 37°C). Samples were collected at 30, 60, 120, 180 and 240 min and immediately analyzed using the HPLC method. Stability was attested when the deviation from the nominal concentrations was within $\pm 15\%$.

Cell viability by MTT assay

Caco-2 cell viability was initially assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. Cells were seeded at a density of $3 \times 10^4 \text{ cells cm}^{-2}$ in a 96-well culture plate containing DMEM media and incubated for 24 h at 37°C in an atmosphere of 5% CO_2 saturation and 90% relative humidity. The medium was removed and $100 \mu\text{L}$ of PD76 solutions at 10, 20 or $40 \mu\text{g mL}^{-1}$ were added in the wells ($n = 8$ for each concentration). The plates were incubated for 12 h at the same conditions. Then, the solutions were carefully removed and replaced with $30 \mu\text{L}$ of MTT (5 mg mL^{-1}) *per* well and incubated again for 2 h. The

supernatant was discarded and the insoluble formazan was dissolved in $70 \mu\text{L}$ of 0.1 M HCl in 2-propanol. Absorbance was determined in a spectrophotometer microplate reader (Thermo Fisher, Waltham, MA, USA) at 570 and 690 nm. DMEM sample was used as the negative control and DMSO:DMEM 1:1 (*v v*⁻¹) was the positive control. The results were expressed as percentage cell viability in relation to the negative control.

Caco-2 cell culture and *in vitro* permeability experiments

Caco-2 cells were purchased from Instituto Adolfo Lutz (São Paulo, SP, Brazil). The cells were cultured in DMEM supplemented with high glucose concentration (4.5 g L^{-1}), 1% non-essential amino acid solution with 10% FBS and 1% glutamine solution at 200 mM. The media was replaced every 48 h and the bottle was stored in an incubator at 37°C , 5% CO_2 saturation and 90% relative humidity.

After reaching $> 80\%$ confluency, cells were trypsinized and seeded at a density of $5 \times 10^4 \text{ cells cm}^{-2}$ in polycarbonate membrane inserts for 12-well plates (Transwell®, $0.4 \mu\text{m}$ pore size). The plates were stored in the incubator, at the same conditions, for 21 days. The transepithelial electrical resistance (TEER) was tested by Millicell ERS® voltameter (Millipore® Corporation, Bedford, MA). TEER values $> 200 \Omega \text{ cm}^2$ were required for conducting the permeability studies.

Both apical to basolateral (A-B) and basolateral to apical (B-A) permeation across Caco-2 cell monolayer were evaluated. Aliquots of PD76 solution at $10 \mu\text{g mL}^{-1}$ were added in the apical chamber (0.5 mL ; $n = 3$) or in basolateral chamber (1.5 mL ; $n = 3$) and the plates were submitted to an orbital shaker at 50 rpm, 37°C . After 0, 30, 60, 120 and 180 min, $200 \mu\text{L}$ of media were collected from each chamber. Replacement of the medium at each time point was performed by adding the same volume of HBSS kept at 37°C . The concentration of PD76 in all collected samples was determined using the developed HPLC method. The correction of the permeated concentration was performed considering the amount of the drug present in the sample volume collected at each time.

In order to evaluate the possible cellular efflux mechanism of PD76, drug permeability was also assessed using a P-glycoprotein (P-gp) inhibitor. Verapamil solutions at $20 \mu\text{g mL}^{-1}$ in HBSS were prepared and added to the plates during 30 min before incubation with PD76 solution.

The A-B and B-A permeability coefficients (P_{app}) were determined based on the following equation 1:

$$P_{\text{app}} = \frac{\text{VR}}{A \times C_0} \times \frac{dC}{dt} \quad (1)$$

where VR is the volume of the receiver chamber (cm^3), C_0 is the initial PD76 concentration in the donor chamber, A is the surface area of Caco-2 monolayer (cm^2) and dC/dt represents the rate of PD76 concentration over time.¹⁵

In silico permeability experiments

The theoretical prediction of PD76 intestinal permeability was performed by means of two computational platforms: ADMETlab 2.0^{16,17} and admetSAR 2.0.^{18,19} The chemical structure of the drug was provided to the platforms in SMILES format, followed by prediction of the permeability in Caco-2 cells and the probability of the drug being substrate for P-gp, related to active efflux process, as well as continuous value of the logarithm of the *n*-octanol/water coefficient partition (Log P). Both platforms employ mathematical models based on chemical structure and/or physicochemical properties of the drug to provide chemical and biological information related to the pharmacokinetic parameters. All *in silico* experiments were statistically validated according to the best practices of QSAR modeling.²⁰ The prediction with at least two different computational methods was done as a consensus prediction strategy²¹ aiming to decrease the error rate and is commonly used in computer-aided drug design. The results found using the *in silico* approach were compared with those obtained by *in vitro* permeability study.

Results

HPLC method development

Initially, the solubility of PD76 was evaluated in HBSS buffer, the diluent used in the permeability study. Due to its low solubility in aqueous solutions, the compound was firstly dissolved in DMSO, using ethanol as co-solvent to prepare the stock solutions. Then, further dilution in HBSS buffer allowed the preparation of working solution at $10.0 \mu\text{g mL}^{-1}$ of PD76. The final concentrations of DMSO and ethanol were 0.5 and 1.1%, respectively, so that these solvents were not harmful to Caco-2 cells.

During the development of the chromatographic method, the mobile phase acidification with formic acid showed to be important to assure adequate PD76 peak shape. Linear gradient elution program was employed aiming to provide proper retention of PD76. The optimized mobile phase was composed of acetonitrile and 2 mM ammonium acetate with 0.1% formic acid, at 1 mL min^{-1} . Detection at 260 nm provided high selectivity and good sensitivity for PD76 and was selected for its detection in permeability experiments (Figure 2).

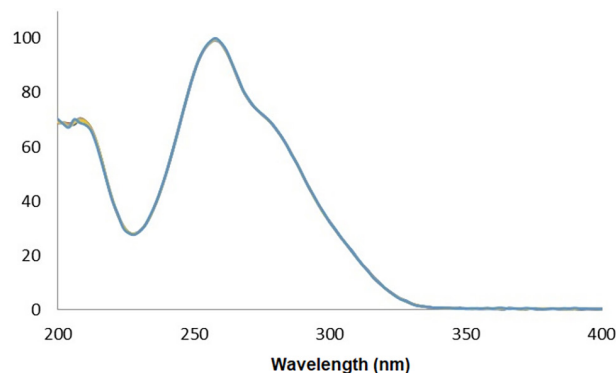


Figure 2. UV spectrum obtained for PD76 solution at $10 \mu\text{g mL}^{-1}$ in HBSS.

Method validation

Selectivity evaluation confirmed that there are no interfering peaks from HBSS and solvents used in the permeability study at the same retention time of PD76 (Figure 3). Purity factors obtained by diode array detector (DAD) were $> 99.9\%$, demonstrating adequate spectral purity of analyte peaks in all assayed sample. No carry-over effect was observed in blank samples analyzed after the injection of PD76 solution at $12 \mu\text{g mL}^{-1}$.

Calibration curves were plotted on three consecutive days and showed to be linear over the range from 0.05 to $12 \mu\text{g mL}^{-1}$. The mean linear regression equation was $y = 236707x - 6231$, with coefficient of determination (r^2) > 0.99 . All back-calculated standard concentrations were within 15% deviation from the nominal value. The residuals had no tendency of variation with concentration. LLOQ obtained for PD76 was $0.05 \mu\text{g mL}^{-1}$, demonstrating the high sensitivity of the developed method.

The intra run and inter run precision and accuracy were calculated by analyzing five replicates of PD76 samples at five concentration levels, in three different days. The obtained RSD and RE values are shown in Table 1. All results meet the acceptable limits of accuracy ($\pm 15\%$) and precision ($\text{RSD} < 15\%$).

During the stability study, PD76 solutions were submitted to the same conditions of permeability study in HBSS media, during 4 h. The obtained RE were 5.48 and 9.66% for 0.1 and $10.0 \mu\text{g mL}^{-1}$ levels, respectively, proving the adequate stability of PD76 at the experimental conditions.

Cell viability determination

Cell viability values were calculated from the exposure of Caco-2 cells to PD76 solutions at different concentrations (10, 20 and $40 \mu\text{g mL}^{-1}$) for 12 h. The cell viability of

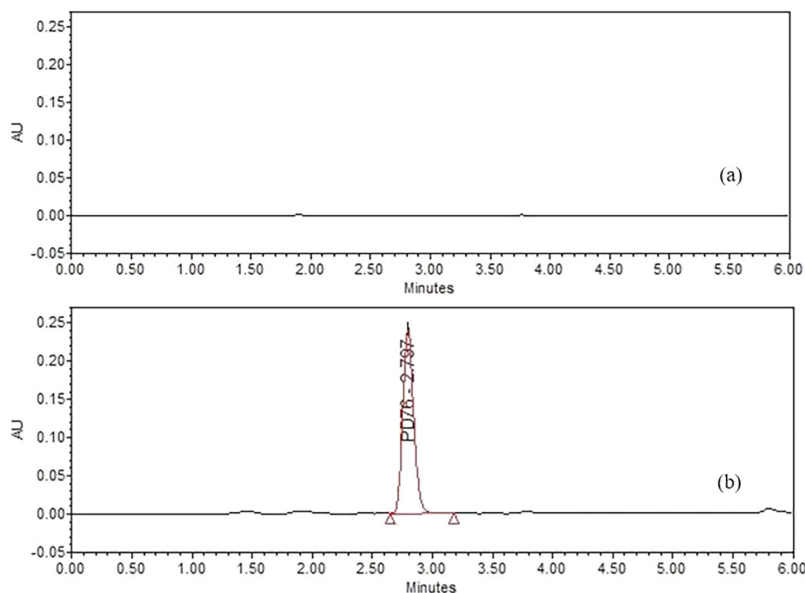


Figure 3. HPLC chromatogram obtained using the developed method for (a) blank solution composed of HBSS and (b) PD76 solution at 10 µg mL⁻¹.

Table 1. Intra run and inter run precision (RSD) and accuracy (RE) for PD76

Concentration / (µg mL ⁻¹)	Intra run (n = 5)		Inter run (n = 15)	
	Precision (RSD) / %	Accuracy (RE) / %	Precision (RSD) / %	Accuracy (RE) / %
0.05	1.71	-2.40	2.00	-1.20
0.10	1.88	9.20	4.49	9.47
5.00	0.28	0.66	0.59	0.75
10.00	1.00	3.12	2.03	1.35
20.00	0.26	0.64	0.25	0.53

RSD: relative standard deviation; RE: relative error.

DMEM sample (negative control) was approximately 95%. The positive control (DMSO:DMEM, 1:1) demonstrated a viability lower than 5%. All PD76 tested concentrations showed to be non-toxic to Caco-2 cells, since the viability values were higher than 70%.²²

In vitro permeability study in Caco-2 cells

Permeability experiments complied with all required assumptions, such as temperature control, rotation, and assurance of membrane integrity by measuring the TEER, which showed values higher than 500 Ω cm² for the inserts of all plates. The recovery rate obtained in the mass balance experiments was close to 90%.

The permeation of PD76 at 10 µg mL⁻¹ in both directions (A-B and B-A) increased linearly with time, up to 180 min (Figure 4). Apparent permeability coefficients (Papp) were calculated and presented in Table 2. Similar experiments were performed using verapamil as P-gp inhibitor and Papp values were also determined.

Table 2. Permeability of PD76 in Caco-2 cells, in the presence and absence of verapamil as P-glycoprotein inhibitor

Permeability coefficients	PD76	PD76 + verapamil
Papp (A-B) / (× 10 ⁻⁶ cm s ⁻¹)	5.25	12.05
Papp (B-A) / (× 10 ⁻⁶ cm s ⁻¹)	23.28	20.12
Efflux ratio (ER): Papp (B-A)/Papp (A-B)	4.43	1.67

Papp: A-B and B-A permeability coefficients. PD76: 2-hydrazinyl-4-(4-methoxyphenyl)thiazole.

In silico permeability study

The validation of *in silico* experiments showed predictability rate higher than 75%.²⁰ For instance, Caco-2 predictive model from ADMETlab presented $r^2 = 0.786$ (n = 2464) and admetSAR presented an accuracy of 76.8% (n = 674). P-gp substrate predictive model from ADMETlab and from admetSAR presented accuracy equal to 84% (n = 1185) and 86.1% (n = 1565), respectively. Lastly, Log P model from ADMETlab presented $r^2 = 0.957$ (n = 12682) and admetSAR used the well-established method AlogP.²³

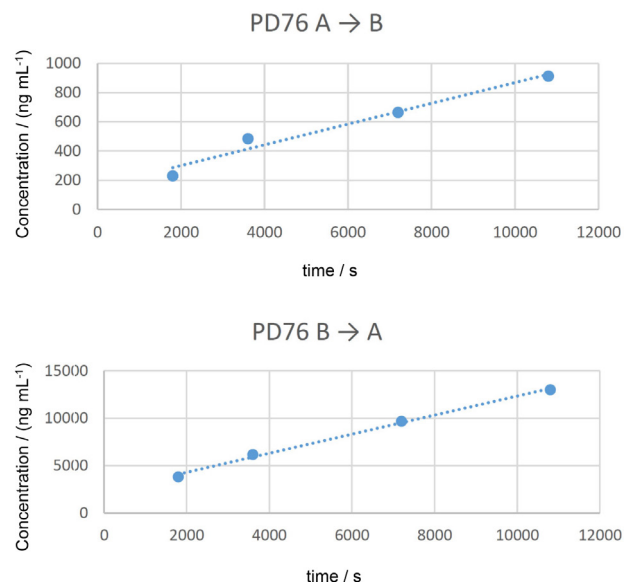


Figure 4. Mean concentrations ($n = 3$) of PD76 permeated across Caco-2 cell membranes over the time in the apical-basolateral (A-B) and basolateral-apical (B-A) directions.

In silico permeability study was carried out for PD76 using ADMETlab and admetSAR platforms, which qualitatively predicted the permeability parameters in Caco-2 cells and the probability of the compound being substrate for P-gp. The Log P value was also calculated indicating that PD76 is in typical range of orally absorbed drugs.²⁴ The obtained results are shown in Table 3.

Table 3. PD76 permeability parameters predicted by ADMETlab and admetSAR platforms

Parameter	ADMETlab	admetSAR
Permeability in Caco-2 cells	+	+
P-gp substrate	+	–
Log P	2.82	2.10

(+): positive prediction; (–): negative prediction; P-gp: P-glycoprotein.

Discussion

MTT assay demonstrated cell viability higher than 70% after exposure of Caco-2 cells to PD76, within acceptable cytotoxic limits.²² Considering that the permeability study was performed with PD76 at $10 \mu\text{g mL}^{-1}$ during 4 h period, the obtained results proved the reliability of the experiments using Caco-2 cell model, since cell damages in the cultured cell monolayer may compromise the permeation and transport functions.

Compounds with Papp (A-B) lower than $1 \times 10^{-6} \text{ cm s}^{-1}$ are considered low permeability drugs; between 1 and $10 \times 10^{-6} \text{ cm s}^{-1}$ are classified as moderately permeable, whereas Papp values above $10 \times 10^{-6} \text{ cm s}^{-1}$ indicate high

permeability drugs.¹⁵ Based on A-B coefficient obtained during the permeability studies, PD76 can be classified as moderately permeable drug. However, the characterization of high or low permeability based on Caco-2 experiments should be complemented by additional studies, as it is widely reported that variability in the results may be found possibly due to cultivation conditions, which can significantly affect the characteristics of the obtained monolayer, as expression of transport and enzymatic proteins.^{25,26} It is worth mentioning that the process of intestinal absorption also depends on the solubility of the compound in the physiological environment. Since PD76 demonstrated poor aqueous solubility in the experiments, additional studies are needed to evaluate the impact of its solubility on the absorption process.

PD76 presented higher permeation in B-A direction, suggesting active efflux transport. This finding has been confirmed by the efflux ratio higher than 2.0, that indicates predominant efflux activity. It is well established that the absorption of some xenobiotics may be decreased by the effect of efflux transporters and P-gp is a prominent protein involved in this mechanism.²⁷ Thus, the same experiments were carried out using verapamil as P-gp inhibitor. A slight decrease in Papp coefficient (B-A) was observed in the presence of verapamil, indicating that PD76 is a probable substrate for P-gp. However, since the efflux ratio remained higher than 1.0, other active efflux transporters may be also involved in PD76 permeation process. In addition, Papp coefficient in apical-basolateral direction increased, demonstrating that the permeation of PD76 across the intestinal epithelium is even higher in the presence of verapamil. The observed reduction in efflux ratio is related to both the decrease of Papp (B-A) and the increase in Papp (A-B). Despite the active efflux process, an adequate intestinal absorption of PD76 can be expected, and consequently, a good oral bioavailability, enabling the clinical administration of this compound by oral route.

The prediction of pharmacodynamic and pharmacokinetic parameters during the drug discovery phase may significantly reduce the failures in clinical studies, besides allowing a faster and less expensive approach. Thus, *in silico* studies have been increasingly used by means of computational models that correlate molecular structures with biological and pharmacokinetic parameters.²⁸ It is well established that some physicochemical properties of drugs, such as molecular size, number of rotatable bonds and Log P are responsible for directing which route the substance will cross the membrane of enterocytes.²⁹ The drug affinity to transporters and enzymes can also be estimated using *in silico* studies, according to some structural properties such as electrostatic potential and lipophilicity.³⁰

According to the *in silico* predictions, ADMETlab and admetSAR platforms inferred that the compound PD76 presents permeability in Caco-2 cells. The results obtained using *in vitro* experiments corroborate these predictions, as they demonstrated that PD76 presents moderate permeability in Caco-2 cells. On the other hand, ADMETlab estimated that PD76 would be a likely substrate for P-gp, contrary to the prediction of admetSAR, which showed a negative result for this process. In fact, *in vitro* studies demonstrated that PD76 may be slightly influenced by the activity of P-gp, leading to active efflux. Since the employed platforms perform only a qualitative prediction, the intensity of the effect cannot be measured using this *in silico* approach. In general, a good agreement between the theoretical and experimental permeability results was obtained for PD76 in our experiments. Specifically, two distinct software were employed and resulted in similar predictions, reinforcing the importance of consensus predictions.³¹ The Log P value calculated by both platforms indicates a compound with adequate lipophilicity, allowing its proper permeation across the intestinal epithelium. The agreement of computational predictions with experimental data reinforces the employment of *in silico* methods in the early stages of drug design accelerating the process and decreasing costs with compounds that could be discarded at the beginning of this phase.

Conclusions

In conclusion, PD76 showed moderate intestinal permeability and the active efflux by P-gp seems to be a probable mechanism related to its oral absorption. Similar results were found by the predictions using *in silico* platforms. Altogether, the results provided useful information for predicting the intestinal permeability of PD76. Further studies are necessary to evaluate the potential of this new antifungal compound for clinical application with administration by oral route.

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Author Contributions

Isabella C. Pierotte was responsible for formal analysis, investigation,

methodology and writing-original draft; Iara R. Silva for the formal analysis, methodology and editing; Valtair S. S. Júnior for formal analysis, investigation and methodology; Gabriel P. Almeida for formal analysis and methodology; Pedro H. G. dos Santos for formal analysis and methodology; Vinícius G. Maltarollo for funding acquisition, supervision and writing-review; Renata B. de Oliveira for conceptualization, funding acquisition, supervision and writing-review; José E. Gonçalves for funding acquisition, investigation, project administration, supervision and writing-review; Isabela C. César for conceptualization, funding acquisition, investigation, project administration, supervision and writing-review.

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