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Caco-2 cells cytotoxicity of nifuroxazide derivatives with potential activity against Methicillin-resistant Staphylococcus aureus (MRSA)

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A B S T R A C T
It is important to determine the toxicity of compounds and co-solvents that are used in cell monolayer permeability studies to increase confidence in the results obtained from these in vitro experiments. This study was designed to evaluate the cytotoxicity of new nifuroxazide derivatives with potential activity against Methicillin-resistant Staphylococcus aureus (MRSA) in Caco-2 cells to select analogues for further in vitro permeability analyses. In this study, nitrofurantoin and nifuroxazide, in addition to 6 furanic and 6 thiophenic nifuroxazide derivatives were tested at 2, 4, 6, 8 and 10 μg/mL. In vitro cytotoxicity assays were performed according to the MTT (methyl tetrazolium) assay protocol described in ISO 10993-5. The viability of treated Caco-2 cells was greater than 83% for all tested nitrofurantoin concentrations, while those treated with nifuroxazide at 2, 4 and 6 μg/mL had viabilities greater than 70%. Treatment with the nifuroxazide analogues resulted in viability values greater than 70% at 2 and 4 μg/mL with the exception of the thiophenic methyl-substituted derivative, which resulted in cell viabilities below 70% at all tested concentrations. Caco-2 cells demonstrated reasonable viability for all nifuroxazide derivatives, except the thiophenic methyl-substituted compound. The former were selected for further permeability studies using Caco-2 cells.

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1. Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is one of the major pathogens responsible for healthcare-associated infections (HA-MRSA) and has also been identified in community-associated infections (CA-MRSA). These bacteria have acquired multidrug resistance to most major antimicrobial classes, including all beta lactams, aminoglycosides, macrolides, fluoroquinolones and tetracyclines, in addition to glycopeptides, such as vancomycin and teicoplanin, which often represent a last resort for therapy (Jones, 2008). However, some vancomycin-resistant strains have recently been described (Oliveira et al., 2001; Walsh and Amies, 2004; Finch and Hunter, 2006; Nordmann et al., 2007). 5-Nitro-heterocyclic compounds with structures similar to nifuroxazide, an antimicrobial agent used to treat gastrointestinal infections, have shown satisfactory activity in vitro against multidrug-resistant strains of S. aureus (Jorge et al., 2009; Masunari and Tavares, 2007). In the course of the development of new drugs, evaluating the bioavailability of lead compounds is necessary to select effective drugs and further improve selected molecular structures.

Caco-2 cells, derived from a human colorectal carcinoma, are the most frequently used of the many in vitro cell culture absorption models. These cells differentiate under culture conditions while retaining most of the morphological and functional properties of human intestinal epithelial cells. Numerous studies have demonstrated that the oral absorption of compounds in humans correlated with their permeability through Caco-2 cells. Therefore, a compound's permeability through these cells is a valuable index used to estimate the uptake of orally administered drugs (Balimane et al., 2000; Gonçalves et al., 2009).

It is important to determine the toxicity of compounds and co-solvents that are used in permeability studies through cell monolayers to increase confidence in the results obtained from these in vitro experiments, as high permeability values can result from cell death and subsequent loss of monolayer function (Blanchfield et al., 2003). There are several standard methods to assess the toxicity of compounds against Caco-2 cells. These assays involve placing the substance in contact with the cultured cells and quantifying the resulting cellular changes by different methods, including the MTT assay protocol described in ISO 10993-5, which is widely used to evaluate the cytotoxicity of new compounds in vitro. This assay is based on the reduction of tetrazolium salts to formazan, a colored product that can be quantified spectrophotometrically.

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The MTT assay (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) is used to quickly and objectively evaluate cell viability based on a colorimetric reaction. In the mitochondria of viable cells, succinate dehydrogenase converts MTT into formazan, a dark blue product that is quantified by colorimetric measurement following cell lysis. As damaged or dead cells exhibit reduced levels of or no dehydrogenase activity, this colorimetric assay of mitochondrial activity can be used to determine cell viability spectrophotometrically (Mosmann, 1983).

The mechanism of toxicity of nitro compounds is associated with the formation of products resulting from the in vivo and in vitro reduction of the nitro groups attached to aromatic rings (Tocher, 1997). Xu et al. (2002) determined that the toxicity of this class of compounds depends on their chemical stability, which is related to the proportion and position of the nitro groups attached to the aromatic rings and directly affects electron delocalization. For example, oxygen is a reactive molecule that promotes the breakdown of many aromatic compounds, which are further metabolized through reactions that are catalyzed by mono- and di-oxygenases (Spain, 1995). However, the strong electron withdrawing character of the nitro groups in poly-nitroaromatic compounds causes an electron deficiency in the aromatic ring, thus favoring initial reduction reactions. The oxidative attack of nitroaromatic compounds occurs under aerobic conditions, while the reductive metabolism of poly-nitroaromatic compounds can occur both aerobically and anaerobically. Thus, the formation of hydroxyl derivatives, which result from the oxidation of the aromatic ring by mono- and di-oxynogenases and subsequent ring opening, represents the mechanistic explanation for the aerobic degradation of mono-nitroaromatic compounds, and some di-nitroaromatic compounds as well (Roldán et al., 1998; Spiess et al., 1998; Zablotowicz et al., 1999; Nishino et al., 2000). Hydroxylamine derivatives that result from the reduction of the nitro group may interact with biomolecules, such as DNA, to cause toxic and mutagenic effects. The toxic effects are related to the electrophilic character of these derivatives, while the mutagenic effects are mainly due to the formation of hydroxylamine adducts resulting from esterification with the nucleic acid guanine. Additionally, some nitroaromatic compounds are intrinsically toxic and function as uncouplers of oxidative phosphorylation, inhibiting the generation of the proton motive force required for ATP synthesis (Roldán et al., 2008).

Currently, there is no data reported on the toxicity of the nitro drugs nifuroxazide and nitrofurantoin in Caco-2 cells, the evaluation of which is necessary prior to conducting permeability tests. This study was designed to evaluate the cytotoxicity of these two drugs and a series of nifuroxazide derivatives with potential activity against MRSA in Caco-2 cells to select analogues for further permeability analyses.

2. Material and methods

2.1. Chemicals

Dulbecco’s modified Eagle medium (DMEM) with and without phenol, penicillin, streptomycin, trypsin–EDTA (0.2%) and fetal calf serum (FCS) were obtained from Cultilab (Campinas, SP, Brazil). Glutamine, d-glucose, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), nifuroxazide (Fig. 1A) and nitrofurantoin (Fig. 1B) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

The 5-nitro-heterocyclic compounds used in this study were designed and synthesized by the Laboratory of Drug Design and Development of the Chemical and Pharmaceutical Technology Department at the Pharmaceutical Sciences Faculty/University of São Paulo. Twelve compounds belonging to an analogous series with structures similar to nifuroxazide were selected (Fig. 2).

2.2. Cell line and culture conditions

The Caco-2 human colon adenocarcinoma cell line (ATCC #HTB-37) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Caco-2 cells were cultured in DMEM supplemented with 4.5 g/L d-glucose (Sigma–Aldrich Corp., St. Louis, MO), NaHCO3 (2.2 g/L) (Sigma–Aldrich Corp., St. Louis, MO), 10% fetal bovine serum (Cultilab, Campinas, SP), 1% non-essential amino acids (Sigma–Aldrich Corp., St. Louis, MO), 100 IU/mL penicillin and 100 µg/mL streptomycin (Cultilab, Campinas, SP) in an atmosphere of 5% CO2 and 90% relative humidity at 37 °C. All cells used in this study were between passages 40 and 50.

2.3. In vitro cytotoxicity

In vitro cytotoxicity assays were performed according to the protocol described in the ISO 10993-5 guide (ISO, 2009). Briefly, Caco-2 cells were cultured in 96-well plates for 24 h at a seeding density of 2 × 104 cells/well. 5-Nitro-heterocyclic compounds were dissolved in dimethylsulfoxide (DMSO), a solvent frequently used when poorly soluble compounds are evaluated in permeability assays, and subsequently diluted in DMEM without phenol to prepare samples of 2, 4, 6, 8 and 10 µg/mL concentration. Four replicates of each concentration for all nitro compounds were assessed in three independent assays for a total of 12 replicates. This range of concentrations was chosen for reasons associated with subsequent permeability tests. First, this range was suggested by prior knowledge related to the absorption of nitrofurantoin, a compound structurally related to nifuroxazide and its derivatives. It is well known that this compound is rapidly absorbed from the small intestine, reaching high plasma concentrations in a few minutes (Buzard et al., 1961). A high permeability rate through Caco-2 cell membranes would be expected for analogous compounds as well, so low initial concentrations would be preferred to avoid sink conditions. Additionally, higher concentrations were prepared, but precipitation was often observed due to the poor solubility of nifuroxazide and its derivatives in Hanks buffer.

Samples containing 2% DMSO (v/v) in DMEM were employed to assess the toxicity of this co-solvent (DMSO control). DMEM samples were used as negative controls, and 50% DMSO (v/v) in DMEM as a positive control. The DMSO and positive controls were evaluated in six replicates and negative controls were evaluated in 12 replicates for each 96-well plate. Experiments were initiated by replacing the culture medium in each well with 100 µL of sample or control solutions, followed by incubation for 4 h at 37 °C in a CO2 incubator. Then, solutions in each well were aspirated, and the cells were incubated again for 2 h with 30 µL of MTT solution (5 mg/mL, Sigma–Aldrich Corp., St. Louis, MO, in DMEM and PBS buffer, 9:1 v/v). Next, formazan crystals were solubilized with 70 µL of isopropyl alcohol acidified with hydrochloric acid and quantified spectrophotometrically at 570 and 690 nm (Spectra Fluor plate reader, Tecan, Austria). Cell viability was calculated based on the measured absorbance relative to the absorbance of the cells exposed to the negative controls, which represented 100% cell viability.

2.4. Statistical analysis

All experiments were performed in 12 replicates (three independent assays, each with four replicates). Data are expressed as means ± standard deviations. Statistical analysis was performed...
than 70% were used as cut-off values. Considered a cytotoxic effect. For this reason, cell viabilities less than 70% were only greater than 70% at 2, 4 and 6 μg/mL, with the latter concentration corresponding to approximately 0.022 mM. A t-test was used to compare nifuroxazide to nitrofurantoin. A one way ANOVA test was applied for multiple comparisons among the nitro compounds and the DMSO controls. For both analyses, means were considered significant if p < 0.05.

### 3. Results

The cytotoxicity of the 5-nitro-heterocyclic compounds in Caco-2 cells in vitro are presented as a percentage of cell viability relative to the negative (100%) and positive controls, as determined using the MTT assay (Fig. 3). The standard deviation was calculated for the 12 replicates of each concentration to determine the variation in cytotoxicity for each compound (Fig. 3). Quantification of cytotoxicity was determined by measuring cell death according to protocols described in ISO 10993-5 (ISO, 2009). This international guide states that a reduction in cell viability by more than 30% is considered apparent differences (p < 0.075) in the cytotoxicity of these drugs at 6 and 8 μg/mL, with the methyl derivative being the most toxic. The methyl-substituted thiophenic derivative (MeTIO) reduced Caco-2 cell viability to less than 70% at all tested concentrations. An ANOVA revealed that only MeTIO had significantly different results at all concentrations evaluated relative to the DMSO control.

### 4. Discussion

To avoid the loss of promising compounds due to insufficient intestinal absorption, adequate screening systems should be implemented at early stages in the drug discovery process to assess and/or predict the ability of a drug to be absorbed. The transport of compounds through cell monolayers is a commonly used method to assess the permeability potential of a compound during the evaluation and selection stages of drug development. The viability of Caco-2 cells treated with the compounds to be used in permeability experiments is a pre-requisite to ensure reliable results. As expected, significant changes in cellular functions, especially those related to cellular transport, are common in cells that have initiated a cell death program. Additionally, the use of co-solvents at high concentrations can affect the integrity of cell membranes. Thus, it becomes essential to assess cell viability in the presence of all substances and at all concentrations that are to be used in permeability assays.

As the 5-nitro-heterocyclic compounds were dissolved in DMSO, it was necessary to evaluate the toxicity of this co-solvent on the cell monolayer at the experimental concentration (2% v/v). The results demonstrated that cell viability for the DMSO control was approximately 90%, which does not preclude its use in further permeability assays at the tested concentration according to ISO.
Furthermore, Da Violante et al. (2002) concluded that DMSO may be used at concentrations up to 10% (v/v) in permeability experiments using Caco-2 cells without any significant cell damage, which supports the results of the present study and the use of this co-solvent in future permeation studies.

The results of the present study indicate that there was no significant toxicity associated with nitrofurantoin and some nifuroxazide derivatives, including AcTIO, EtoTIO, BrTIO and AminoTIO, at the tested concentrations. However, significant toxicity, as indicated by cell viabilities less than 70%, was observed for nifuroxazide concentrations greater than 0.03 mM (8 \mu g/mL) following incubation for 4 h. Blumenstiel et al. (1999), using nitrofuran derivatives with activity against \textit{Trypanosoma cruzi}, demonstrated that nifuroxazide and nifuprazine had no toxic effects on mammalian cells (macrophages) following incubation for 3 days at concentrations lower than 10 mM. In a cytotoxicity study conducted by Rossa et al. (2003), the nitrofuran quinifuril showed high toxicity in rat leukemic cells in vitro and low toxicity in non-tumor animal cells. Therefore, it is plausible that the toxicity of nitro compounds is dependent on multiple factors, including chemical structure, exposure time, dosage and the type of cell. Caco-2 cells were more susceptible to 5-nitro-heterocyclic compounds than were macrophages (Blumenstiel et al., 1999), probably because the former are derived from a carcinoma, i.e., they are mammalian tumor cells. As these cytotoxic assays were performed prior to their cellular maturation into epithelial cells, it is expected that these cells would have greater viability when they are differentiated. As treatment with the majority of the compounds, except for the MeTIO derivative, resulted in cell viabilities greater than 70% at 2 and 4 \mu g/mL, the latter was selected for further analysis of Caco-2 cell permeability.

Treatment with the methyl-substituted thiophenic derivative (MeTIO) resulted in Caco-2 cell viabilities of less than 70% at all tested concentrations; therefore, it was removed from the 5-nitro-heterocyclic series of compounds that were further evaluated for permeability. However, it is noteworthy that the methyl-substituted furanic derivative (MeFUR) was more toxic than the methyl-substituted thiophenic derivative at 6, 8 and 10 \mu g/mL. With the exception of these compounds, Caco-2 cell viability was greater for cells treated with the thiophenic derivatives relative to their respective furanic compounds. This result can be explained by the higher atomic radius of the sulfur atom relative to the oxygen atom. Consequently, the electrons in the valence shell of the former, which is the outermost layer of electrons and is responsible for the atom’s reactivity, are spread over a larger space, resulting

![Fig. 3. Viability of Caco-2 cells incubated for 4 h with: (A) nitrofurazide and nitrofurantoin, (B) thiophenic derivatives and (C) furanic derivatives, all assessed by the MTT technique. Each of the five concentrations was evaluated in 12 replicates for each compound. Error bars depict the primary results based on standard deviation. The red line separates the cell viability values above and below 70%. Nitrofurantoin was the least toxic substance against Caco-2 cells. The methyl-substituted thiophenic derivative (MeTIO) was the only substance which showed cell viability values less than 70% for all tested concentrations. *A t-test was used to compare nifuroxazide to nitrofurantoin; a one way ANOVA test was applied for multiple comparisons among the nitro compounds and the DMSO controls. For both analysis, means were considered significant if \( p < 0.05. \) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
in higher stability and lower reactivity than the oxygen atom. Thus, the greater cytotoxicity of the furanic derivatives could be associated with the oxygen atom, which, due to its greater electron donor characteristics, would increase the reactivity of the nitro group attached to the furan ring. The differences in cytotoxicity between furanic and thiophene derivatives relative to their molecular volume and surface charge can be explained based on differences in their electrostatic and lipophilic properties, as illustrated in Fig. 4. Fig. 4a depicts the electron density surface of BrTIO, the least toxic compound of the series, and BrFUR, showing the greater radius of the sulfur atom and the higher electron density in the oxygen atom. Fig. 4b illustrates the surface electrostatic potential of BrTIO and BrFUR, which indicates that the most reactive portion of the molecule is larger in the furanic derivatives compared to the thiophene derivatives. The toxicity of the MeTIO compound could be related to the increased hydrophobicity that is conferred to the molecule by the methyl group, as all other substituents are more hydrophilic in character. Thus, the cytotoxicity of these nifuroxazide derivatives could be influenced by both the reactivity of the ring atom linked to the nitro group and the hydrophobicity of the substituent on the benzene ring.

In addition to the cytotoxic evaluation of the substances that will be used in further permeability assays, we performed an analysis of Caco-2 cell monolayer integrity. Transepithelial electrical resistance (TEER) is extensively utilized as an indicator of cell viability (Hidalgo, 1996), in combination with the use of model drugs, i.e., compounds with known permeability values (FDA, 2000). These latter two methods will be used in the further evaluation of the nifuroxazide derivatives permeability. By comparing the results from these analyses with the cytotoxic data, we can increase the confidence in the permeability data obtained.

In addition to the use of Caco-2 cells as a screening tool for the assessment of intestinal permeability, these cells have also been used to predict the toxicity of new oral drug candidates and formulation adjuvants in epithelial cells (Konsoula and Barile, 2005; Shah et al., 2004; Rhoads et al., 2010). Consequently, we could conclude that the MeTIO derivative is highly toxic to the intestinal epithelium, which would invalidate its use as a drug. However, the AcTIO, EtoTIO, BrTIO and AminoTIO derivatives displayed lower toxicity than did nifuroxazide itself, which would make them potential new drug candidates to be used in the treatment of MRSA strains.

**Conflict of interest statement**

There is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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