



Development of a sensitive HPLC method to measure *in vitro* permeability of *E*- and *Z*-isomeric forms of thiosemicarbazones in Caco-2 monolayers

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ABSTRACT

In the current study, we developed a HPLC method to quantitatively measure the permeability of the BpT-based chelators, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT) and 2-benzoylpyridine 4-allyl-3-thiosemicarbazone (Bp4aT), across human colorectal adenocarcinoma (Caco-2) monolayers as a model of gut absorption. In aqueous solution, Bp4eT and Bp4aT formed inter-convertible *Z* and *E* isomers that were resolved by HPLC. Peak area was linear with respect to chelator concentration. Acceptable within-day and between-day precision (<22%) and accuracy (85–115% of true values) were obtained over a range of 1.0–100 μ M for Bp4eT and 1.5–300 μ M for Bp4aT. Limits of detection were 0.3 μ M and 1 μ M for Bp4eT and Bp4aT, respectively, while corresponding limits of quantification were 1 μ M and 5 μ M. Both chelators showed significant ability to chelate iron in THP-1 cells using a calcein-based assay and no apparent cytotoxicity was observed within 24 h. Ratios of the apical to basolateral and basolateral to apical transport for Bp4eT were 1.10 and 0.89 at 100 μ M and 300 μ M respectively, indicating equal bi-directional movement of the compounds. Similarly, ratios were 0.77 and 0.92 for Bp4aT, respectively. This study demonstrates that Bp4eT and Bp4aT can be efficiently transported through Caco-2 cells and can potentially be formulated for oral delivery.

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1. Introduction

Thiosemicarbazone compounds are chelators that bind a variety of transition metals, including iron (Fe), copper (Cu), and zinc (Zn) [1,2]. The di-2-pyridylketone thiosemicarbazone (DpT) group of thiosemicarbazones (Fig. 1) possesses potent and selective anti-tumor activity [3]. Recently, 2-benzoylpyridine (BpT) analogs were synthesized from the DpT series by replacing one of the 2-pyridyl groups of the DpT compounds with a phenyl ring (Fig. 1) [4]. The presence of the 2-benzoylpyridine moiety within the BpT compounds enhanced the redox activity of their Fe complexes and also enhanced their lipophilicity, iron mobilization efficacy and ability to inhibit iron uptake from the iron transport protein, transferrin

[4]. Overall, members of the DpT and BpT series of compounds are highly effective iron chelators in cell culture [3].

Human immunodeficiency virus-1 (HIV-1) infection is associated with progressive iron deposition in bone marrow, liver, brain and other organs [5]. The higher levels of iron in these tissues have a number of effects, including: (1) enhancement of HIV-1 replication, in which iron plays a role in the synthesis of viral particles by HIV-infected host cells [6–8]; (2) facilitation of the growth of bacterial invaders, for which iron serves as an important nutrient [9]; (3) depression of granulocyte, macrophage and lymphocyte function and potential promotion of neoplastic growth [10,11]; and (4) down-regulation of stress protein production [12]. Therefore, iron chelators which reduce iron levels may have a dual benefit in HIV-1 therapeutic intervention by both relieving the effects of HIV-1-induced iron overload and directly inhibiting HIV replication.

Several studies have reported the potential of iron chelators to act as HIV-1 replication inhibitors [7,13]. Previously, we reported the inhibition of HIV-1 transcription in cultured cells by the iron chelators, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311; Fig. 1) and 4-[3,5-bis-(hydroxyphenyl)-1,2,4-triazol-1-yl]-benzoic acid (ICL670, or desferasirox; Fig. 1), the latter of which

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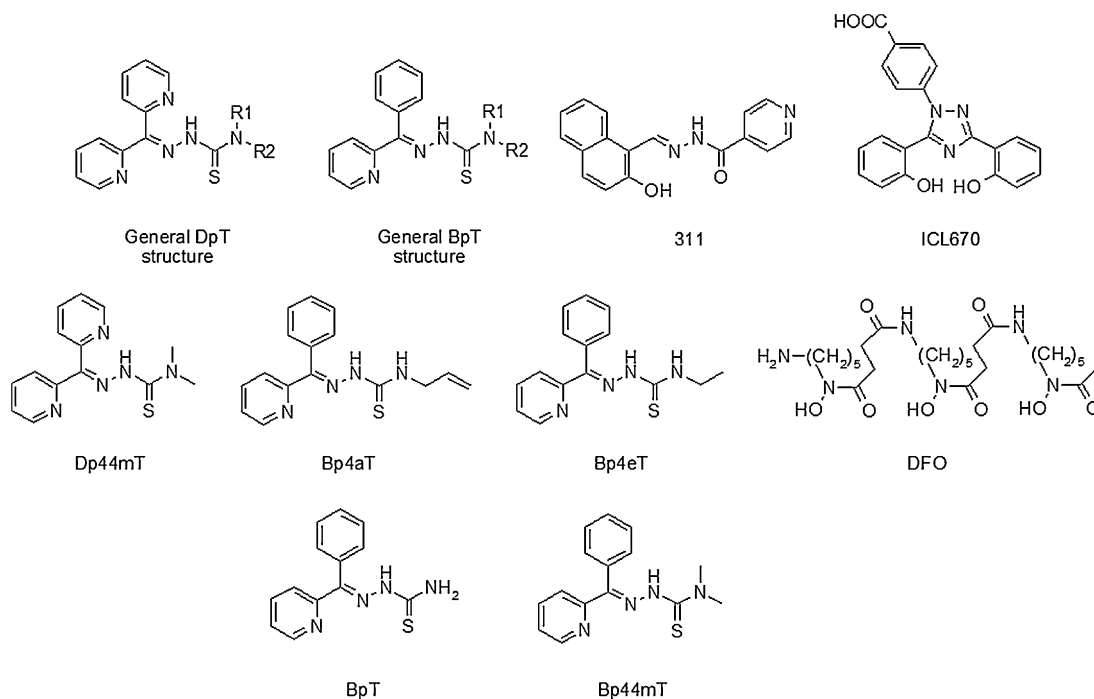


Fig. 1. Structures of the chelators discussed in this article. General structure of the DpT analogs; general structure of the BpT analogs; 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone (311); 4-[3,5-bis-(hydroxyphenyl)-1,2,4-triazol-1-yl]-benzoic acid (ICL670); di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT); 2-benzoylpyridine 4-allyl-3-thiosemicarbazone (Bp4aT); 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT); desferrioxamine (DFO); 2-benzoylpyridine thiosemicarbazone (BpT); and 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone (Bp44mT).

is an FDA-approved orally administered iron chelator currently in clinical use [14]. Recently, we showed that the BpT chelators inhibit HIV-1 transcription and viral replication *in vitro* in cultured T cells and primary peripheral blood mononuclear cells (PBMCs) [15]. In this previous study, we screened a library of BpT- and DpT-based iron chelators for their ability to inhibit HIV-1 transcription and identified di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1), 2-benzoylpyridine 4-allyl-3-thiosemicarbazone (Bp4aT; Fig. 1) and 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT; Fig. 1) as the most potent inhibitors. We demonstrated that these compounds down-regulate the cellular activity of cyclin-dependent kinases-2 and -9 (CDK-2 and CDK-9), both of which are involved in the regulation of HIV-1 transcription [16]. Based on these promising data, we concluded that these novel compounds may have potential use in anti-retroviral regimens and may be useful as components of combination therapy.

The most widely used iron chelator on the market today is desferrioxamine (DFO; Fig. 1), which has been in clinical use for over 40 years for the treatment of iron overload. Though clinically effective, DFO has the drawback of having relatively high hydrophilicity, which reduces its ability to cross the lipid bilayer of cell membranes [17] and lowers its potential for absorption from the gastrointestinal tract. Consequently, the drug must be given parenterally, as a continuous subcutaneous infusion, or as a continuous intravenous infusion over long periods of time (12–24 h/day; 5–6 days/week) [18,19]. On the other hand, ICL670 is absorbed more effectively by the intestine and also has a longer half-life than DFO [20]. Unfortunately, ICL670 was less than optimal in its inhibition of HIV-1 replication, with an IC_{50} value in the high micromolar range [14]. Thus, our focus has shifted to evaluating the therapeutic effectiveness of the new chelators, Bp4eT and Bp4aT. To this end, in the studies reported herein, we sought to investigate the cellular permeability of Bp4eT and Bp4aT as an initial indicator for assessing how they are absorbed from the gut. First, we developed and

validated a HPLC method to measure the levels of the *E* and *Z* isomeric forms of Bp4eT and Bp4aT in transport media. Second, we employed this method to: (1) determine the iron chelating properties of the compounds in cultured THP-1 cells; (2) measure their cytotoxicity; and (3) assess their permeability across Caco-2 monolayers.

2. Materials and methods

2.1. Chemicals and materials

Acetic acid, ammonium acetate, ethylenediaminetetraacetic acid (EDTA) and HPLC-grade acetonitrile and water were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.2. Preparation and analysis of chelator solutions

The DpT- and BpT-series chelators used in this study were synthesized and characterized by standard methods in our laboratory [4]. Stock solutions were prepared in DMSO at 100 mM concentrations. Chelator solutions were prepared by making further dilutions in Hank's Balanced Salt Solution (HBSS) from the stock solution. The final DMSO concentration was 0.5% in the 500 μ M preparation which was the highest concentration used in the cytotoxicity study. In addition, the final DMSO concentration was 0.3% in the 300 μ M preparation which is the highest concentration used in the transport study. After dilution, the chelator solutions were then ultrasonicated to ensure complete solubility. The solutions were warmed to 37 °C prior to the transport study or prior to injection into the HPLC system to prevent precipitation. The stability of the chelator solutions was tested under the following storage conditions: (i) 4 °C for one week with more than one freeze–thaw; (ii) room temperature for 4 h and (iii) 40 °C for 4 h. Sample solutions were prepared in the range of 10–200 μ g/mL.

2.3. Chromatographic separations

The HPLC system employed included a Waters 2695 Alliance peristaltic pump and a Waters 996D UV detector (Waters Corp., Milford, MA). Separations were carried out on a reversed-phase Discovery HS F5 column (250 mm × 4.0 mm × 5 μm, Sigma–Aldrich). The column was kept at 40 °C with a flow rate of 1 mL/min. The mobile phase was composed of 2 mM ammonium acetate/10 mM EDTA buffer and acetonitrile (50:50, v/v) at pH 5.8. The UV detector wavelength was set at 318 nm and 20 μL samples were injected in all experiments. 10 μM Dp44mT was used as an internal standard. The limit of detection (LOD) of the method was defined as the lowest concentration of an analyte that gave a signal-to-noise ratio of 3:1. On the other hand, the limit of quantitation (LOQ) was defined as the minimum concentration of the analyte that can be estimated quantitatively with suitable precision and accuracy. The linearity of the method was evaluated using standard solutions at five different concentrations of each of the chelators within the range of 1–150 μM in quadruplicate. Mean peak area values were plotted against the corresponding concentrations. Within-day (intra-day) precision was determined from the analysis of six individually prepared chelator standard solutions in the range of 1–300 μM. The same measurement at three different concentrations (15, 30, and 100 μM) was repeated over the course of the next five days to determine between-day (inter-day) precision. Accuracy is defined in the literature as an estimation of the nearness of test data procured by a method to the true value. Method accuracy was estimated based on the calculated recovery of the analyte from prepared solutions compared to blank samples. The HPLC data were analyzed using Waters Breeze HPLC software (Waters Corp.).

2.4. Iron chelation assays

The iron chelation assay we employed involved comparing the effects of the various chelators on the labile iron pool (LIP) in human acute monocytic leukemia (THP-1) cells. We followed the protocol of Cabantchik and colleagues [21] who saturated cells with calcein, a weak iron chelator, the fluorescence of which is quenched upon binding iron. A cell-permeable derivative of calcein, namely calcein-AM, was added to washed THP-1 cells. Once inside the cells, calcein-AM was cleaved by endogenous intracellular esterases to give calcein, which bound the available iron. Treatment of the cells with iron chelators removed the calcein-bound iron, releasing the calcein and causing an increase in its fluorescence; this was a measure of the amount of cellular iron that had been effectively removed by the iron chelators [21]. We used the following formula to analyze the data: $F/F_i = 1 + k(Q)$, where F_i is the fluorescence in the presence of quencher (iron) at time 0; F is the fluorescence at a given time after the addition of chelator; and Q is the concentration of the quencher (iron). Thus, the value of $(F - F_i)/F_i$ is proportional to the concentration of chelated iron when equilibrium has been reached and the calcein fluorescence has been de-quenched. This latter index is a measure of the chelator's activity in cells.

2.5. Preparation of cell monolayer cultures

Human epithelial colorectal adenocarcinoma (Caco-2) cells (HTB-37, passage 18) used in this study were purchased from American Type Culture Collection (ATCC; Manassas, VA) and were cultured in complete Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1% glutamine and 1% each of the antibiotics, penicillin and streptomycin, at 37 °C in a 5% CO₂ atmosphere. Cells were passaged at 80–90% confluency using 0.05% trypsin–EDTA and the medium was changed every other day. For the permeability study, cells were cultured as monolayers in 12-well polycarbonate transwell inserts

(with a pore size of 0.4 μm and a diameter of 12 mm) obtained from Corning–Costar (Corning, NY) at a density of 62,000 cells/cm².

2.6. Measurement of labile iron

The labile iron pool (LIP) was analyzed following the protocol of Cabantchik and colleagues, where an incubation with calcein-AM, followed by treatment with the iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH), was used to detect the amount of chelatable cellular iron [21]. THP-1 cells were treated with 25 μM ferric sulfate for 1 h and then supplemented with 0.2 μM calcein-AM (Invitrogen/Molecular Probes) for 10 min at 37 °C. The cells were then washed with PBS, resuspended in PBS and placed in 96-well plates. Then the iron chelators, 10 μM Bp4eT, 10 μM Bp4aT or 30 μM SIH, were added and fluorescence was immediately measured using the Light Cycler 480 (Roche) which was programmed for continuous cycles of 2 min incubation at 37 °C and then fluorescent measurement in the SYBR Green I detection format (excitation at 465 nm and emission at 510 nm). The first measurement was designated as zero measurement. As with the iron chelation assay described above, we used the formula: $F/F_i = 1 + k(Q)$. Similarly, the value of $(F - F_i)/F_i$ was proportional to the concentration of chelator when equilibrium was reached and calcein fluorescence was quenched.

2.7. Cytotoxicity assay

Caco-2 cells were grown in 96-well plates at 37 °C and incubated with the iron chelators at concentrations of up to 500 μM for 24 h at 37 °C. To assess cytotoxicity, media was removed and the cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) to remove any extracellular serum esterase activity that might interfere with the assay (see below). We then used the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen/Molecular Probes; Cat#: L3224) which utilizes a standard protocol implementing calcein-AM. Cells were supplemented with 0.2 μM calcein-AM (Molecular Probes, Invitrogen), a compound that, when hydrolyzed by intracellular esterase activity, releases the fluorescent, iron-binding compound, calcein. The cells were incubated with calcein-AM for 10 min at 37 °C. Fluorescence intensity, which was proportional to the number of viable cells present, was measured using a model LS50B Luminescence Spectrometer (PerkinElmer, Waltham, MA) equipped with a robotic 96-well plate scanner using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. A positive control containing 100% dead cells was prepared by treating the cells with Triton X-100 (1%, v/v), incubating them with calcein-AM at 0.2 μM, and measuring fluorescence intensity.

2.8. Drug transport (permeability) assays

The movement of Bp4eT and Bp4aT across Caco-2 monolayers was studied by adapting a published protocol [22] with minor changes. Briefly, Caco-2 cells were grown on 24-well transwell plates containing DMEM complete media, which was changed every other day until cells became totally confluent and a monolayer was evident. The transport study was performed on the twenty-first day after initiation of the growth of the cells. The Caco-2 cell monolayers were washed twice with HBSS containing 20 mM HEPES and then treated with one or the other of the iron chelators at concentrations of 100 and 300 μM. Chelators were added to the apical (AP) or basolateral (BL) wells in 0.3 mL or 0.6 mL of transport buffer for bidirectional transport evaluation. After incubating the culture at 37 °C for 30, 60 and 120 min, 100 μL aliquots were removed from each receiving chamber and replaced with the same volume of transport buffer to maintain experimental homeostasis.

The aliquots were analyzed by HPLC in quadruplicate. The apparent permeability coefficients (P_{app}), expressed in cm/s, were calculated by using the following equation [23]:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{60} \times \frac{1}{A} \times \frac{1}{C_0}$$

where $\Delta Q/\Delta t$ is the permeability rate (nmol/min) and is the same as the slope of the plot of the amount (in nmol) of chelator traversing the monolayer versus time (in min); A is the surface area of the cell membrane (cm^2); and C_0 is the initial concentration of chelator in the donor chamber (μM).

2.9. Lucifer yellow (LY) permeability assay

The lucifer yellow (LY) permeability assay was performed both at the beginning and at the end of the transport study. The purpose of this assay was to test the integrity of the Caco-2 monolayers used for the drug transport investigations, described above. In this assay, LY, a fluorescent compound, was placed on one side of the monolayer, and fluorescence was measured on the opposite side. An absence of fluorescence on the opposite side was indicative of a tightly sealed, intact monolayer. Briefly, Caco-2 cells grown on 12-well transwell plates were washed three times with HBSS. Then, 300 μL of LY solution (100 $\mu\text{g}/\text{mL}$) was added to each well in the donor tray and 600 μL of HBSS was added to each well in the receiver tray for the apical-to-basolateral (AP-to-BL) transport study. For the basolateral-to-apical (BL-to-AP) transport study, the converse was conducted: 600 μL of LY solution (100 $\mu\text{g}/\text{mL}$) was added to each well in the donor tray and 300 μL of HBSS was added to each well in the receiver tray. The culture plate was incubated for 2 h at 37 °C and 100 μL aliquots were collected and transferred to a 96-well plate for measurement of fluorescence intensity. Excitation and emission wavelengths were set at 485 nm and 535 nm, respectively. In this manner, the extent of “leakage” through the monolayer was assessed.

2.10. Statistical analysis

The program, Statistical Package for the Social Sciences (SPSS) version 11.0, procured from IBM Corp. (Somers, NY), was employed for statistical analysis. Results are presented as mean \pm standard deviation ($n=3$ determinations) in a typical experiment of 3 performed. Data were compared using Student's t -test. Results were considered significant when $p < 0.05$.

3. Results

3.1. Development and validation of an HPLC method to measure Bp4eT and Bp4aT levels

We developed and validated a HPLC method with UV detection to measure levels of Bp4eT and Bp4aT, and have applied this protocol to chelation and transport (permeability) studies. A method originally developed to measure Dp44mT [24], a thiosemicarbazone with a structure similar to those of Bp4eT and Bp4aT (Fig. 1), served as a starting point for our method development and validation protocols. Chromatographic separations were achieved by using a reversed-phase column with the mobile phase composed of a mixture of ammonium acetate buffer and acetonitrile (62:38, v/v). The temperature of the system was maintained at 40 °C. The mobile phase buffer pH was found to be optimal at pH 5.8, both in terms of the solubility of the compounds and peak resolution. The method we developed enabled the successful separation of Bp4eT, Bp4aT and Dp44mT, the latter of which was employed as an internal standard (IS) for the study.

Both Bp4eT and Bp4aT were eluted as two separate peaks, and we identified these peaks as corresponding to the *E* and *Z* isomeric forms of the compounds (Fig. 2). This assignment was based on a study by Stariat et al. [25] that used an LC-MS/MS method to demonstrate that Bp4eT exists in the *E* and *Z* isomeric forms. Under the chromatographic conditions employed, the retention times were 6.8 min and 12.0 min for Bp4eT (Figs. 2B and C); 7.5 min and 13.2 min for Bp4aT (Figs. 2D and E); and 3.75 min for the IS, Dp44mT (Figs. 2B and C). The combined (*E* plus *Z*) peak area of Bp4eT was several-fold greater than that of Bp4aT (compare Figs. 2B and D), despite the fact that equal amounts of each were loaded onto the column. This indicates that the extinction coefficient at 318 nm is higher for Bp4eT than Bp4aT.

The HPLC data also showed that immediately after solution preparation from the powder form, the majority of each compound existed predominantly as the *Z* isomer (Figs. 2B and D). However, a major *Z*-to-*E* shift was observed after 24 h at 4 °C (Figs. 2C and E). Therefore, for validation of the HPLC method and for the drug transport studies, sample solutions were prepared in HBSS and stored at 4 °C for a minimum of one day before they were used. This temperature and buffer condition resulted in the same peaks for the two isomers at the same concentration. Therefore, data analysis was performed using the ratio of the sum of the peak areas of the two isomers to the area of the IS compound, Dp44mT. No major interfering peaks were observed for either the compounds in HBSS or HBSS buffer alone (blank). Furthermore, the size of the peak corresponding to Dp44mT (IS) was not affected by the presence of the chelators (data not shown).

Calibration curves were linear over the concentration range of 1.5–100 μM for Bp4eT and 5–150 μM for Bp4aT as shown in the supplemental data (Fig. S1). The mean ($n=3$) correlation coefficients for the linear curves were 0.9941 ± 0.0016 for Bp4eT and 0.9981 ± 0.0001 for Bp4aT. Mean y -intercepts were 0.0328 ± 0.0014 ($n=3$) and 0.0406 ± 0.0028 ($n=3$) for Bp4eT and Bp4aT, respectively. The mean slopes ($n=3$) were 0.0514 ± 0.0019 for Bp4eT and 0.0129 ± 0.0003 for Bp4aT.

The LODs for Bp4eT and Bp4aT, which are the minimum concentrations of chelator that give a signal-to-noise ratio of 3, were 0.3 μM and 1.0 μM , respectively. The LOQs, which are the minimum concentrations that can be determined with acceptable precision (CV < 20%), were 1.0 μM for Bp4eT and 5.0 μM for Bp4aT. Assay precision was within an acceptable level in terms of both same-day (<10% CV) and between-day (<22% CV) variation within the linear range of 1.0–100 μM for Bp4eT and 5.0–150 μM for Bp4aT. The precision (and accuracy) data are shown in Table 1. Since there are no commercially available standards for the two compounds, we adapted the published International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline for estimation of accuracy [26]. The method accuracy was derived from the linearity, precision and specificity data. Specificity data is based on the comparison of calculated recovery of the analyte from prepared solutions to the blank samples. This approach was employed considering the high purity of the compounds. The two chelators in HBSS solution were stable over a 5 day period when stored at 4 °C with a precision of CV < 22% (Table 1 – presented as interday precision data). Further, the chelators were stable for 4 h at room temperature (25 °C) and 40 °C (data not shown).

3.2. Iron chelating properties of Bp4eT and Bp4aT

The chelation properties of Bp4eT and Bp4aT were determined using a calcein-based method. For this assay, we used cultured promonocytic THP-1 cells, which have some of the characteristics of human macrophages [27], the type of cells that consume aged red blood cells and play a critical role in recycling or storing

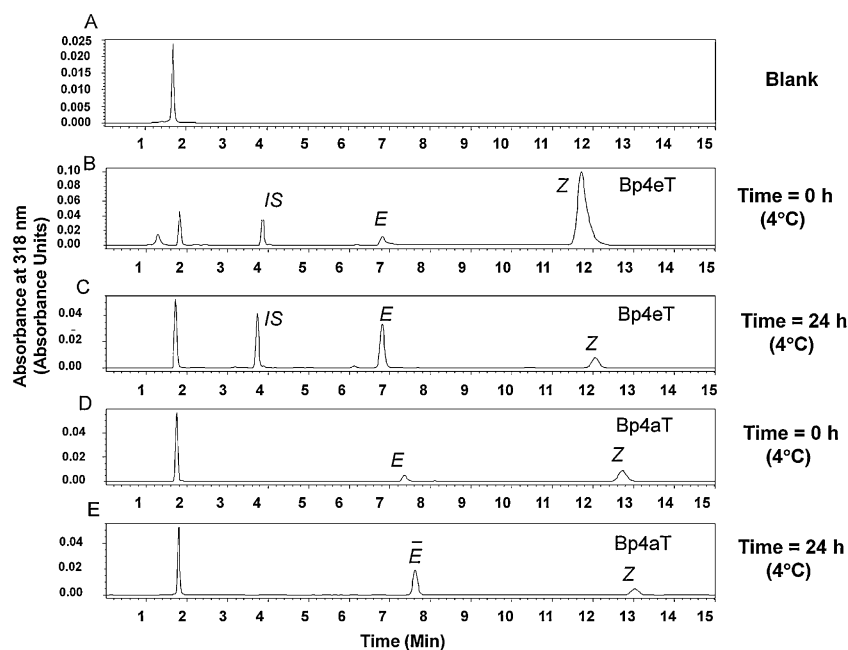


Fig. 2. Representative chromatograms showing Z-to-E isomer conversion over time for Bp4eT and Bp4aT in aqueous solution. (A) Blank HBSS buffer; (B) 50 μ M Bp4eT and 10 μ M Dp44mT in HBSS buffer (20 μ L) injected immediately after preparation of the solution from the solid; (C) under the same conditions as (B), but after a 24 h incubation at 4 $^{\circ}$ C; (D) 50 μ M Bp4aT and 10 μ M Dp44mT in HBSS buffer (20 μ L) injected immediately after preparation from the solid; and (E) under the same conditions as (C), but after a 24 h incubation at 4 $^{\circ}$ C. IS, internal standard, Dp44mT. Results are a typical experiment of three performed.

the iron derived from the breakdown of hemoglobin. The THP-1 cells were loaded with ferric sulfate (25 μ M) and then treated with non-fluorescent calcein-AM, which, after being transported into the cells, is converted to calcein, a weak iron-binding fluorescent compound, the fluorescence of which is quenched upon binding to iron. After addition of iron chelators to the THP-1 cells pre-treated with calcein-AM, an increase in the fluorescence was observed as the calcein released its iron to the added chelator, which binds iron with considerably higher affinity. Thus, the iron chelating ability of experimental compounds was measured. We compared the chelating abilities of Bp4eT and Bp4aT to the well-studied aroylhydrazone chelator, SIH, which was used as a positive control [28]. The results (Fig. 3) show that both Bp4aT and Bp4eT are slightly more effective intracellular iron chelators than SIH, binding higher levels of

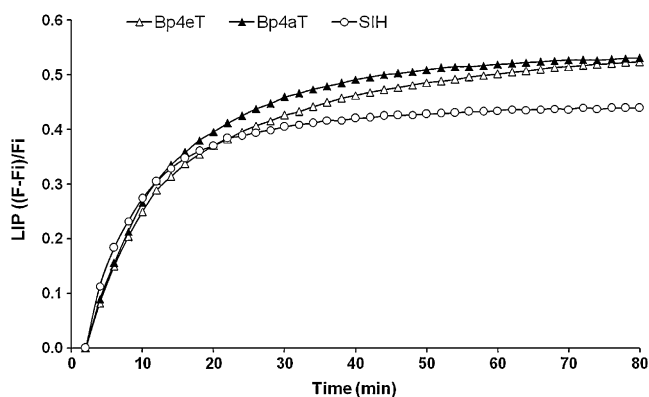


Fig. 3. Cellular iron chelation by Bp4eT and Bp4aT using THP-1 cells in culture. THP-1 cells were treated with iron(II) sulfate (10 μ M) for 3 h at 37 $^{\circ}$ C. After this, the cells were loaded with calcein-AM (0.2 μ M) for 10 min at 37 $^{\circ}$ C, and then 10 μ M of the indicated iron chelator was added. Fluorescence intensity of calcein was measured in real time starting just after the addition of chelator. Here, the size of the labile iron pool (LIP) in the cells is represented as fractional fluorescence $(F - F_1)/F_1$, where F_1 is fluorescence in the presence of quencher at time 0, and F is fluorescence at a given time. LIP is plotted against time of incubation with chelator (in min). Results are means of 3 determinations in a typical experiment of three performed.

iron from the so-called LIP. Thus, both Bp4eT and Bp4aT showed significant ability to chelate intracellular iron in cultured cells.

3.3. Cytotoxicity of short-term Bp4eT and Bp4aT incubations with Caco-2 cells

We then analyzed the effect of Bp4eT and Bp4aT on the normal growth of Caco-2 cells over 24 h to determine whether any toxic effects would be evident during the transport experiments. The results show that both Bp4eT and Bp4aT had little effect on Caco-2 cell growth at concentrations up to 500 μ M after 24 h of treatment, although the ligand BpT, which shows low anti-proliferative activity [29] did have some effect, with cell viability dropping to around 70% (relative to control) at 500 μ M BpT (Fig. 4).

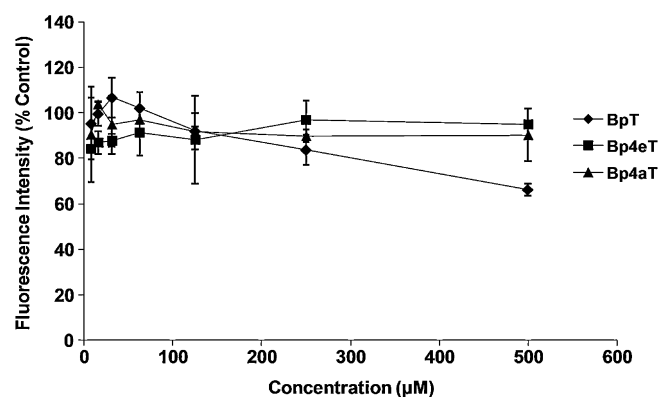


Fig. 4. Effect of the chelators Bp4eT, Bp4aT and BpT on the growth of Caco-2 cells. Caco-2 cells were grown on 96 well plates (2×10^5 cells/well) and treated either of the chelators for 24 h/37 $^{\circ}$ C. Then the cells were loaded with 0.2 μ M calcein-AM for 10 min/37 $^{\circ}$ C and fluorescence was measured. Fluorescence intensity of the treated cells was compared to the untreated (control) cells and expressed as % of Control Fluorescence Intensity for the chelators, Bp4eT, Bp4aT, and also BpT, a chelator with low anti-proliferative activity. Fluorescence intensity (% control) was plotted against concentration (in μ M). Results are mean \pm SD (3 determinations) in a typical experiment of three experiments performed.

Table 1
Accuracy and Precision of the HPLC method for the analysis of the iron chelators, Bp4eT, Bp4aT and Dp44mT, in the transport buffer (HBSS).

Theoretical concentration (μM)	Peak area (mean \pm S.D.)	CV (%)	No. of samples analyzed
Bp4eT			
<i>Intra-day assay</i>			
0.0	1433 \pm 0.0	0.0	
0.5	5468 \pm 284	5.2	4
1.5	13,744 \pm 528	3.8	4
5	84,977 \pm 3744	4.4	4
15	257,376 \pm 3191	1.2	4
50	620,703 \pm 18,182	1.8	4
100	1,081,750 \pm 20,019	1.9	4
<i>Inter-day assay</i>			
15	228,054 \pm 49,715.8	21.8	3
30	600,321 \pm 91,849.11	15.3	3
100	1,093,472 \pm 137,777.5	12.6	3
Bp4aT			
<i>Intra-day assay</i>			
0.0	39 \pm 0.0	0.0	
1	985 \pm 93	9.4	4
3	3801 \pm 974	8.6	4
10	17,983 \pm 1034	5.8	4
30	78,047 \pm 1851	2.4	4
100	401,998 \pm 9390	2.3	4
300	535,005 \pm 1275	0.2	4
<i>Inter-day assay</i>			
15	30,872 \pm 3859	12.5	3
30	60,974 \pm 6463	10.6	3
100	512,615 \pm 47,160	9.2	3
Dp44mT			
<i>Intra-day assay</i>			
0.0	263 \pm 0.0	4.9	4
0.3	1511 \pm 71	4.7	4
1	3641 \pm 191	5.2	4
3	14,790 \pm 1419	9.6	4
10	154,297 \pm 10,171	6.6	4
30	598,872 \pm 35,040	5.9	4
100	2,132,558 \pm 29,299	1.4	4
300	6,352,227 \pm 62,680	1.0	4
1000	20,322,660 \pm 270,978	1.3	4

The two chelators in HBSS solution were stable over a 5 day period when stored at 4 °C with a precision of CV < 22%, as shown in the table. Results are mean \pm SD ($n = 3$).

3.4. Transport of Bp4eT and Bp4aT across Caco-2 cell monolayers

3.4.1. The integrity of the monolayer

The integrity of the junctions between the Caco-2 monolayers and the drug transport transwell plate walls was evaluated using lucifer yellow (LY) permeability assays. If a monolayer forms a tight seal against the plate wall, then leakage of the fluorescent LY through (or around) it will be negligible. The assays were conducted prior to, and after, each drug transport experiment. Our data showed no significant LY infiltration, confirming the integrity of the monolayer (Table 2). Less than 2% of LY was recovered from

Table 2
Measurement of Caco-2 monolayer integrity by LY leakage.

Chelator added: Bp4aT	% LY leakage	Chelator added: Bp4eT	% LY leakage
100 μM		100 μM	
AP-to-BL	0.55 \pm 0.1	AP-to-BL	0.52 \pm 0.1
BL-to-AP	0.79 \pm 0.2	BL-to-AP	1.82 \pm 0.1
300 μM		300 μM	
AP-to-BL	0.52 \pm 0.1	AP-to-BL	0.49 \pm 0.1
BL-to-AP	0.77 \pm 0.3	BL-to-AP	1.1 \pm 0.31

Results are mean \pm SD ($n = 3$).

Abbreviations: AP, apical; BL, basolateral.

the receiver chambers for transport in either direction (apical-to-basolateral or basolateral-to-apical) at the ends of the experiments.

3.4.2. Transport kinetics of Bp4eT and Bp4aT in Caco-2 monolayers

The transport/movement of Bp4eT and Bp4aT across the monolayer was measured in both apical (AP)-to-basolateral (BL) and BL-to-AP directions. A bi-directional transport study helps to avoid potential bias due to back-diffusion of the drugs from the receiving chamber. The time course of transport of the compounds at 100 μM or 300 μM in both directions is shown in Fig. 5. At 30 min, the compounds were detectable in the receiving chamber in both directions. The transport of each compound in both directions increased pseudo-linearly with increasing time of incubation. The data also showed that the transport of Bp4aT was faster than that of Bp4eT by approximately 1.5-fold. The P_{app} values calculated from the slopes of the curves shown in Fig. 5 are summarized in Table 3. There was no significant difference between the rates of AP-to-BL and BL-to-AP transport for both chelators (Fig. 5 and Table 3). The ratio (R_{net}) of the P_{app} values for the two directions of transport was near unity (0.8–1.1; Table 3), indicating that there is no inherent directional bias for the transport.

4. Discussion

Treatment of chronic illnesses, such as HIV-1, requires long-term drug administration. For this reason, oral dosing is the method of choice for treatment. In considering the development of iron chelators as a long-term treatment in combination therapy, it is therefore critical to assess the suitability of the compounds for oral therapy. Drug absorption is a key pharmacokinetic parameter in oral drug therapy. The “gold-standard” iron chelating agent, DFO, has very poor absorption through the gastrointestinal tract and must be administered parenterally [30]. Although an alternative, ICL670 (Deferasirox), is a well-absorbed and -tolerated orally effective iron chelator, its inhibition of HIV-1 replication is less than optimal. In fact, the IC_{50} value of ICL670 is at high micromolar concentrations as opposed to the nanomolar concentrations seen with Bp4eT and Bp4aT [14,15]. Therefore, we were interested in studying the oral permeability potential of the latter two iron chelators. In order to perform these drug transport studies, a reliable analytical method to measure the chelators was critical. HPLC is a widely used technique for the analysis of compounds and has become a primary method for measurement of non-volatile small molecules [31]. We chose to develop a HPLC method, with a focus on its practical applications to future pre-clinical and clinical studies.

Here we have developed a simple and reliable HPLC method for the determination of Bp4eT and Bp4aT in drug transport buffer. Preliminary UV spectrophotometric scans of the compounds revealed a maximum absorption at 318 nm. Therefore, UV detection at this wavelength was used for all HPLC experiments. The compound Dp44mT was selected as the HPLC internal standard due to its structural similarity to the BpT series compounds (Fig. 1), combined with its significantly different retention time (see Fig. 2). Our validation data indicate that the method is sensitive and reliable in our transport studies. The developed procedure allowed acceptable separation and quantification of the compounds we analyzed, with a run time of less than 15 min. It provided acceptable accuracy (85–115% of true values) and precision (CV < 22% in both intra- and inter-day assays). The LOQ values were < 1 μM for both compounds, which were found to be stable in drug transport buffer for several days at 4 °C. Therefore, the HPLC method developed can be generally suitable for future studies examining Bp4eT and Bp4aT.

Both Bp4eT and Bp4aT showed no apparent cytotoxicity on Caco-2 cells at concentrations of $\leq 500 \mu\text{M}$ over 24 h (Fig. 4), unlike

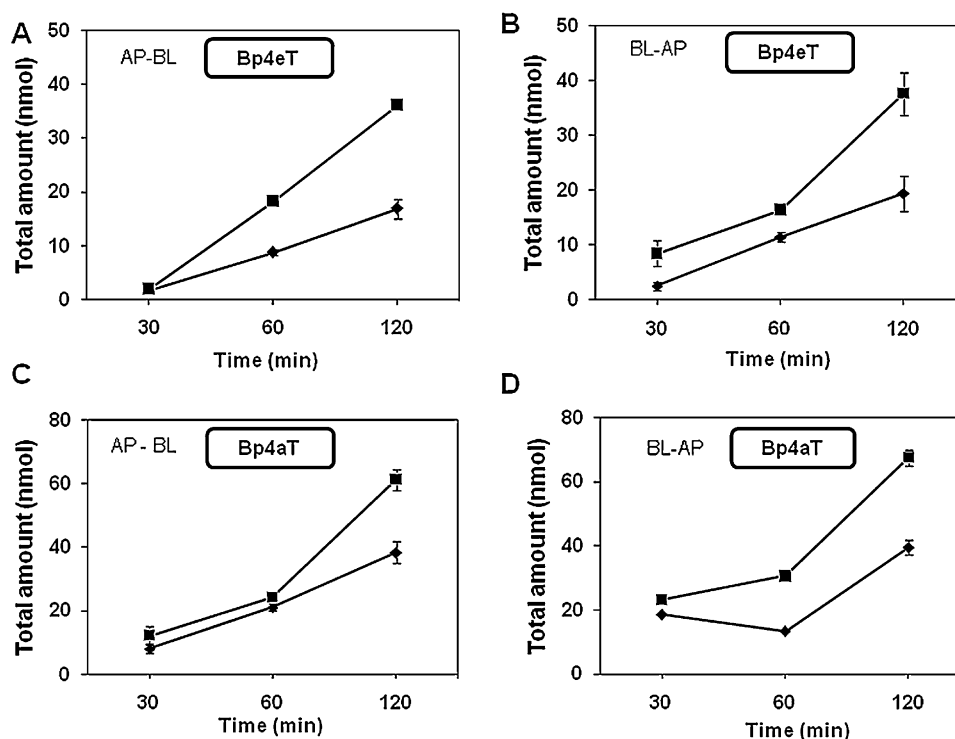


Fig. 5. Transport of Bp4eT and Bp4aT across Caco-2 monolayers. Bp4eT or Bp4aT was loaded onto either the apical (AP) or the basolateral (BL) side of Caco-2 monolayers at the indicated concentrations and incubated at 37 °C for the indicated times (30, 60 and 120 min). Samples were collected from the receiving side and the total amount of chelator present was measured by HPLC. Total amount measured (in nmol) is plotted against time of incubation. (A, B) Bp4eT; (C, D) Bp4aT. *Solid triangles*: 100 μM of chelator; *solid squares*, 300 μM of chelator. Results are mean \pm SD (3 determinations) in a typical experiment of three experiments performed.

human embryonic kidney (HEK)-293T and the HIV-1-susceptible CEM cells, where the DpT and BpT series of chelators demonstrated anti-proliferative activity [15]. Therefore, we used two concentrations of these ligands, 100 μM and 300 μM , for the transport studies. Given the results of the cytotoxicity study, these concentrations should not be toxic to Caco-2 cells, especially since the incubation time (2 h) used for the transport study was much shorter than the 24 h incubation used for the cytotoxicity assay. Moreover, the observation that cell monolayers remained intact and did not become 'leaky' during the course of these experiments also attests to a lack of cytotoxicity by these chelators.

Our transport studies showed that both Bp4eT and Bp4aT are permeable in Caco-2 monolayers and have permeability values similar to those for other oral drugs [32]. For example, ICL670 has been reported to have a P_{app} value of $1.66 \pm 0.13 \times 10^{-5}$ cm/s for Caco-2 cells *in vitro* [33], which is similar to the values of 1.7×10^{-5} cm/s and 2.6×10^{-5} cm/s we report here for Bp4eT and Bp4aT, respectively. Also, the data herein did not show much directional bias in transport, whether from AP-to-BL or from BL-to-AP, as indicated by R_{net} values which were close to 1.0 for both compounds at different concentrations. Since, the BpT chelators are novel compounds, their interactions with various drug efflux transporters such as P-glycoprotein (Pgp), multidrug

resistance associated protein (MRP) and breast cancer resistance protein (BCRP) are yet to be determined. Indeed, their carrier-mediated influx, metabolism and protein-binding properties have yet to be characterized. Hence, we assumed linearity in the uptake/efflux conditions to provide the best possible approximation of P_{app} values in the Caco-2 monolayers. In the future, the Catenary model developed by Sun et al., may be potentially useful to employ to further examine permeability under non-linear conditions [34].

Bp4aT had a relatively higher permeability in Caco-2 monolayers compared to Bp4eT (Fig. 5). The two compounds have similar structures (Fig. 1) and molecular weights. However, our calcein-based chelation experiments seem to indicate that Bp4aT is a more efficient iron chelating agent than Bp4eT. Significantly, both Bp4aT and Bp4eT sequester cellular iron by binding it through the same type of coordination site [4]. Thus, the reason for their different iron chelation activities is not apparent. It should be recognized that the higher rate of cellular permeability of Bp4aT relative to Bp4eT (Fig. 5) seen in the assay may have contributed to the higher apparent chelation efficacy of Bp4aT that was observed (see Table 3).

Data from our previous studies showed that, among the series of chelators that we tested [14,15], Bp4eT and Bp4aT were the most effective in terms of inhibition of HIV-1 transcription. The

Table 3

Apparent permeability (P_{app}) values for Bp4eT and Bp4aT. The P_{app} values were calculated from the slopes of the curves in Fig. 5 (Ref. [22]). R_{net} is the ratio of P_{app} (BL-to-AP) to P_{app} (AP-to-BL).

Conc. (μM)	Bp4eT			Bp4aT		
	P_{app} ($\times 10^{-5}$ cm/s) AP-to-BL	P_{app} ($\times 10^{-5}$ cm/s) BL-to-AP	R_{net}	P_{app} ($\times 10^{-5}$ cm/s) AP-to-BL	P_{app} ($\times 10^{-5}$ cm/s) BL-to-AP	R_{net}
100	0.809 ± 0.119	0.889 ± 0.196	1.10	1.670 ± 0.016	1.292 ± 0.282	0.77
300	1.819 ± 0.302	1.627 ± 0.107	0.89	2.756 ± 0.261	2.534 ± 0.454	0.92

Results are mean \pm SD ($n = 3$).

Abbreviations: AP, apical; BL, basolateral.

transport data presented here suggest the BpT chelators should demonstrate good intestinal absorption. Of relevance, assessment of the oral activity of these compounds using “Lipinski’s Rule of 5” which considers characteristics such as the octanol–water partition coefficient, the number of H-bond donors/acceptors and the molecular mass, showed these agents had properties enabling absorption after oral administration [2]. The potential for oral administration of these agents has also been illustrated by recent studies demonstrating that the BpT series analog, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone (Bp44mT; see Fig. 1), showed marked efficacy against lung cancer xenografts in mice when given *via* the oral route [35]. Collectively, these data are very encouraging for future development of oral formulations of these compounds. In addition, it is likely that further structural modifications of Bp4eT and Bp4aT could lead to the discovery of even better chelators for treatment of HIV-1 infection or cancer.

5. Conclusions

In conclusion, we have developed a simple and sensitive HPLC method to detect and measure levels of the novel iron chelators, Bp4eT and Bp4aT, which are shown to have good permeability in Caco-2 cells. Our results demonstrating enhanced permeability and little or no cytotoxicity, at least over short periods of incubation (2–24 h), suggest that Bp4eT and Bp4aT could potentially be formulated for oral therapy. Our data, described both here and in our previous studies, provide evidence that Bp4eT and Bp4aT have an acceptable therapeutic window, a novel mechanism of action, and a desirable drug permeability profile. Thus, they deserve further investigation in terms of their use in future anti-HIV-1 treatment or anti-tumor regimens. Our findings are consistent with the possibility that these iron chelators may be used in oral anti-retroviral combination therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2012.08.011>.

References

- [1] S.N. Pandeya, D. Sriram, G. Nath, E. DeClercq, *Eur. J. Pharm. Sci.* 9 (1999) 25.
- [2] Y. Yu, D.S. Kalinowski, Z. Kovacevic, A.R. Siafakas, P.J. Jansson, C. Stefani, D.B. Lovejoy, P.C. Sharpe, P.V. Bernhardt, D.R. Richardson, *J. Med. Chem.* 52 (2009) 5271.
- [3] P.J. Jansson, P.C. Sharpe, P.V. Bernhardt, D.R. Richardson, *J. Med. Chem.* 53 (2010) 5759.
- [4] D.S. Kalinowski, Y. Yu, P.C. Sharpe, M. Islam, Y.T. Liao, D.B. Lovejoy, N. Kumar, P.V. Bernhardt, D.R. Richardson, *J. Med. Chem.* 50 (2007) 3716.
- [5] C. de Monye, D.S. Karcher, J.R. Boelaert, V.R. Gordeuk, *AIDS* 13 (1999) 375.
- [6] G.J. Kontoghiorghes, E.D. Weinberg, *Blood Rev.* 9 (1995) 33.
- [7] M. Hoque, H.M. Hanauske-Abel, P. Palumbo, D. Saxena, D. D’Alliessi Gandolfi, M.H. Park, T. Pe’ery, M.B. Mathews, *Retrovirology* 6 (2009) 90.
- [8] N.A. Georgiou, T. van der Bruggen, M. Oudshoorn, H.S. Nottet, J.J. Marx, B.S. van Asbeck, *J. Infect. Dis.* 181 (2000) 484.
- [9] E.D. Weinberg, *J. Eukaryot. Microbiol.* 46 (1999) 231.
- [10] J.R. Boelaert, G.A. Weinberg, E.D. Weinberg, *Infect. Agents Dis.* 5 (1996) 36.
- [11] G.A. Weinberg, H. Friis, J.R. Boelaert, E.D. Weinberg, *Clin. Infect. Dis.* 33 (2001) 2098.
- [12] P. Lipinski, R.R. Starzynski, A. Stys, M. Stracilo, *Postepy Biochem.* 56 (2010) 305.
- [13] B.S. van Asbeck, N.A. Georgiou, T. van der Bruggen, M. Oudshoorn, H.S. Nottet, J.J. Marx, *J. Clin. Virol.* 20 (2001) 141.
- [14] Z. Debebe, T. Ammosova, M. Jerebtsova, J. Kurantsin-Mills, X. Niu, S. Charles, D.R. Richardson, P.E. Ray, V.R. Gordeuk, S. Nekhai, *Virology* 367 (2007) 324.
- [15] Z. Debebe, T. Ammosova, D. Breuer, D.B. Lovejoy, D.S. Kalinowski, K. Kumar, M. Jerebtsova, P. Ray, F. Kashanchi, V.R. Gordeuk, D.R. Richardson, S. Nekhai, *Mol. Pharmacol.* 79 (2011) 185.
- [16] S. Nekhai, K.-T. Jeang, *Future Microbiol.* 1 (2006) 417.
- [17] K.O. Hamilton, L. Stallibrass, I. Hassan, Y. Jin, C. Halleux, M. Mackay, *Br. J. Haematol.* 86 (1994) 851.
- [18] F. Di Gregorio, M.A. Romeo, G. Pizzarelli, G. Aiello, G. Russo, *Br. J. Haematol.* 98 (1997) 601.
- [19] B. Cooper, H.F. Bunn, R.D. Propper, D.G. Nathan, D.S. Rosenthal, W.C. Moloney, *Am. J. Med.* 63 (1977) 958.
- [20] R. Galanello, A. Piga, D. Alberti, M.C. Rouan, H. Bigler, R. Sechaud, *J. Clin. Pharmacol.* 43 (2003) 565.
- [21] S. Epsztejn, O. Kakhlon, H. Glickstein, W. Breuer, I. Cabantchik, *Anal. Biochem.* 248 (1997) 31.
- [22] S. Zhou, X. Feng, P. Kestell, B.C. Baguley, J.W. Paxton, *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* 809 (2004) 87.
- [23] F. Tang, R.T. Borchardt, *Pharm. Res.* 19 (2002) 787.
- [24] J. Stariat, P. Kovarikova, J. Klimes, D.B. Lovejoy, D.S. Kalinowski, D.R. Richardson, *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* 877 (2009) 316.
- [25] J. Stariat, P. Kovarikova, J. Klimes, D.S. Kalinowski, D.R. Richardson, *Anal. Bioanal. Chem.* 397 (2010) 161.
- [26] ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1), 4 (2005) 9.
- [27] A. Asseffa, L.A. Dickson, S. Mohla, T.A. Bremner, *Oncol. Res.* 5 (1993) 11.
- [28] T. Simunek, C. Boer, R.A. Bouwman, R. Vlasblom, A.M. Versteilen, M. Sterba, V. Gersl, R. Hrdina, P. Ponka, J.J. de Lange, W.J. Paulus, R.J. Musters, *J. Mol. Cell. Cardiol.* 39 (2005) 345.
- [29] D.S. Kalinowski, P.C. Sharpe, P.V. Bernhardt, D.R. Richardson, *J. Med. Chem.* 50 (2007) 6212.
- [30] T. Toliyat, M. Jorjani, Z. Khorasanirad, *Drug Deliv.* 16 (2009) 416.
- [31] S. Bansal, A. DeStefano, *AAPS J.* 9 (2007) E109.
- [32] S. Yamashita, T. Furubayashi, M. Kataoka, T. Sakane, H. Sezaki, H. Tokuda, *Eur. J. Pharm. Sci.* 10 (2000) 195.
- [33] X.P. Huang, M. Spino, J.J. Thiessen, *Pharm. Res.* 23 (2006) 280.
- [34] H. Sun, K.S. Pang, *Drug Metab. Dispos.* 36 (2008) 102.
- [35] Y. Yu, Y. Suryo Rahmanto, D.R. Richardson, *Br. J. Pharmacol.* 165 (2012) 148.