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Hemolytic potential of miltefosine is dependent on cell concentration: Implications for in vitro cell cytotoxicity assays and pharmacokinetic data

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ABSTRACT

Miltefosine possesses antiparasitic, antibacterial, antifungal and antitumor activities; however, its mechanism of action is not well established. In the current work, the miltefosine concentrations required to achieve 50% hemolysis in PBS were shown to vary from 600 μM using 5×10^9 cells/mL to ~2.9 μM for ~5×10^6 cells/mL. This cell concentration-dependent hemolytic potential was described using an equation that included the membrane–water partition coefficient (K) and miltefosine concentrations in the cell membrane (c_m) and aqueous medium (c_w) as variables. The best-fit values for the 50% hemolysis data were log K = 4.68, c_m = 110.8 mM, and c_w = 2.3 μM. Hemolysis measurements in whole blood were used to determine the erythrocyte membrane–plasma partition coefficient of miltefosine (Log $K_{M/P}$ = 1.77). Additionally, miltefosine concentration in whole blood was found to be ~86% of that in plasma. Previously reported clinical pharmacokinetics data indicate that the plasma concentration of miltefosine peaks at ~90 μg/mL when treating visceral leishmaniasis. Using this concentration, which corresponds to ~77 μg/mL miltefosine in whole blood, we found only 2.8% hemolysis. Significant hemolysis (5.4%) was observed only after doubling the concentration to 180 μg/mL. Recently reported data indicate that miltefosine inhibitory concentrations in *Leishmania* are also dependent on cell concentration. The biophysical parameters assessed in the current study indicated that this type of response is associated with the accumulation of the drug in the cell membrane, which becomes damaged when critical drug concentrations are reached.

1. Introduction

Miltefosine was the first oral drug approved for the treatment of visceral, cutaneous and mucocutaneous leishmaniasis. It is currently approved for leishmaniasis treatment in India (2002), Germany (2004), Colombia (2005) and the United States (2014, in patients aged ≥12 years) [1]. Miltefosine is also used as a topical treatment for breast cancer skin metastases and cutaneous lymphoma [2]. Following oral administration, edelfosine, an analog of miltefosine, shows strong activities against mantle cell lymphoma and chronic lymphocytic leukemia in xenograft mouse models and preferentially accumulates in tumors [3]. Miltefosine also exhibits antibacterial [4] and broad-spectrum antifungal activities [5–7].

Miltefosine has been reported to possess similar modes of action in *Leishmania* parasites and human cancer cells. Its activity is mainly associated with inhibition of phospholipid turnover and lipid-dependent cell signaling pathways; this inhibition results in apoptosis [2,8–11]. Using electron paramagnetic resonance (EPR) spectroscopy, miltefosine was recently demonstrated to dramatically increase lipid and protein dynamics in *Leishmania* [12] and erythrocyte [13] membranes and extracellular lipid dynamics in the stratum corneum [14], the uppermost skin layer. Miltefosine does not alter lipid fluidity in model membranes [14,15]; as such, it appears to preferentially act on membrane proteins [13,14].

Typical EPR experiments require high cell concentrations in the capillaries that contain samples (minimum of 5×10^8 cells/mL to obtain good EPR spectra). Because of this, EPR spectroscopy can only be used to detect changes in cell membranes for high miltefosine concentrations. These concentrations are higher than the hemolytic concentrations used for experiments with low hematocrit levels or the inhibitory miltefosine concentrations typically found in antiproliferative assays. Indeed, it is generally believed that the antiproliferative or antitumor activities of miltefosine occur at lower, more clinically relevant concentrations and that miltefosine only causes cell lysis at higher concentrations [9].

Importantly, the miltefosine inhibitory concentration required to combat *Leishmania* increases with increasing cell concentration [12]. The aims of the current study were to elucidate the biophysical

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parameters associated with the relationship between drug cytotoxic capacity and the concentration of cells in suspension and to investigate how these parameters affect the results of antiproliferation assays. In addition, we assessed whether miltefosine-induced changes in *Leishmania* cell membranes, which are detectable by EPR spectroscopy at high cell concentrations, are consistent with the drug concentrations that inhibit cell growth at lower cell concentrations. To accomplish this, we analyzed the relationship between miltefosine hemolytic potential and hematocrit levels. Hemolysis measurements in whole blood were used to delineate the association between miltefosine concentrations in blood plasma and those in whole blood.

2. Materials and methods

2.1. Hemolytic potential of miltefosine in PBS

Blood was donated by laboratory researchers and placed into EDTA-coated blood collection tubes under a vacuum. One volume of blood was diluted in three volumes of PBS and centrifuged at 2000 × *g* for 10 min at 4 °C. Three additional centrifugations were performed; after each, plasma and white blood cells were carefully removed by aspiration, and the pellet was resuspended in PBS. Miltefosine (Avanti Polar Lipids Inc., Alabaster, USA) was diluted in PBS to 2 mg/mL (homogeneous suspension), and 100 μL samples were prepared of varying hematocrit and miltefosine concentrations. After incubation for 2 h at 25 ± 1 °C, 1.4 mL PBS was added to each tube, and the samples were centrifuged again. The percentage of hemolysis was determined based on the absorbance of hemoglobin in the supernatant at 540 nm using the following equation:

$$\%\text{hemolysis} = \frac{A_S - A_{Cl}}{A_{C2} - A_{Cl}}, \tag{1}$$

where A_S represents a sample containing the desired miltefosine concentration, A_{C1} is a control sample without miltefosine, and A_{C2} is a fully hemolyzed sample in deionized water.

2.2. Hemolytic potential of miltefosine in whole blood

Blood was centrifuged at 2000 × *g* for 10 min at 4 °C, and one part plasma (corresponding to half of the blood volume) was collected. Miltefosine was diluted in the plasma at twice the desired concentration and then added to the other half of the blood sample. The samples were placed into closed tubes and incubated for 48 h at 5 ± 1 °C; during this period, the samples were gently stirred several times. Experiments were also conducted with incubations of 24 h at 5 ± 1 °C and 2 h at 37 ± 1 °C. Hemolysis percentages were determined as described above (Eq. (1)). The only exception was the calculation of the absorbance A_{C2}, for which a high concentration of miltefosine (5 mg/mL) was used to obtain 100% hemolysis.

3. Results

Fig. 1 shows the hemolysis curves of miltefosine for several concentrations of erythrocytes in PBS. The miltefosine concentration causing 50% hemolysis varied considerably with the concentration of cells in suspension. To better understand the dependence of hemolysis produced by a hydrophobic molecule on cell concentration, several biophysical parameters of the cell suspension were taken into consideration in this work. Identification of the biophysical parameters affecting our results is not only relevant to hemolysis experiments but also to any trial that involves interactions of drugs with cells.

For hydrophobic molecules in cell suspensions, molar concentration cannot be calculated in the usual manner. To examine this calculation in more detail, consider a cell suspension with n_w moles of a test molecule

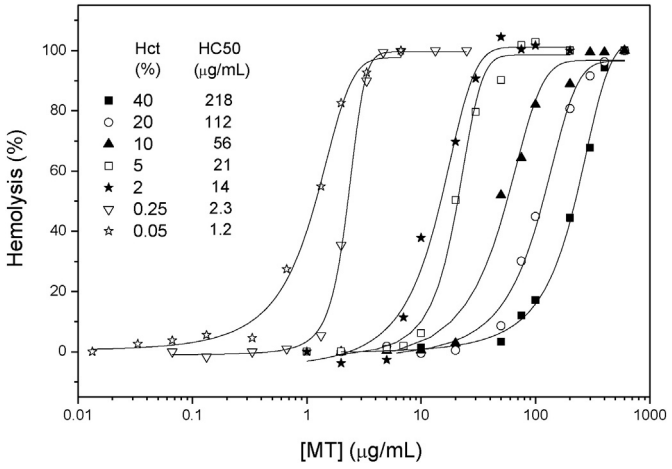

Fig. 1. Hemolysis curves of miltefosine in PBS at different hematocrit (Hct) levels. The miltefosine concentrations required for 50% hemolysis (HC50) are listed.

in aqueous medium and n_m moles in cell membranes. The molar concentration in the suspension (c_{sus}) is given by.

$$C_{sus} = \frac{n_w + n_m}{V_w + V_m}, \tag{2}$$

where V_w is the aqueous medium volume, and V_m is the membrane volume of the cells in suspension. Rewriting Eq. (2) in terms of molar concentrations in the membranes, c_m, and the aqueous medium, c_w, and introducing the equilibrium constant, described as the mole-fraction partition constant or membrane–water partition coefficient K, where $K = c_m / c_w$, leads to the following:

$$C_{sus} = \frac{c_w V_w + K c_w V_m}{V_w + V_m}, \tag{3}$$

or

$$\frac{C_{sus}}{c_w} = \frac{(V_w/V_m) + K}{(V_w/V_m) + 1}. \tag{4}$$

If $V_w \gg V_m$, then Eq. (4) indicates that $c_{sus} = c_w$, i.e., the calculated concentration is accurate for well-diluted systems. If the test molecule is hydrophilic, the value of K is small, and again $c_{sus} = c_w$, indicating that the calculation is also accurate in this case. However, for higher cell concentrations and hydrophobic molecules, the suspension can no longer be considered homogeneous, and c_{sus} can be quite different from c_w.

In the graph shown in Fig. 1, the miltefosine concentrations in cell suspension are expressed as c_{sus}. Fig. 2a shows a plot of the miltefosine concentration (c_{sus}) that produces 50% hemolysis (Fig. 1) as a function of the calculated V_w/V_m in the erythrocyte suspension. The best-fit curve using Eq. (4) gives estimated values of K and c_w in the erythrocyte suspension. To determine the V_w/V_m values in the abscissa of the plot from Fig. 2a, we performed an initial estimate using 40% hematocrit (this value can also be adjusted to obtain the best-fit curve). The other volumetric ratio data were calculated from the initial value assumed for 40% hematocrit. In these calculations, the relationship with other hematocrit levels used in the experiments was maintained (e.g., the V_w/V_m value for 20% hematocrit was twice that for 40%).

To estimate V_w/V_m, we used a mean erythrocyte volume of 88 fL [16], an average erythrocyte surface area of 135 μm^2 [17] and an erythrocyte membrane thickness of 78 Å [18,19]. By multiplying the area of an erythrocyte by the thickness of its membrane, its membrane volume can be obtained ($V_m = 1.05 \times 10^{-12}$ mL). V_m is 83.8 times smaller than the erythrocyte volume (88×10^{-12} mL); thus, $V_w/V_m = 83.8$ for a