Unbound Ritonavir Concentrations in Rat and Human Hepatocytes

JANNEKE KEEMINK, PATRICK AUGUSTIJNS, PIETER ANNAERT

Drug Delivery and Disposition, KU Leuven Department of Pharmaceutical and Pharmacological Sciences, Leuven, Belgium

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ABSTRACT: Knowledge regarding intracellular drug exposure is crucial to gain mechanistic understanding of hepatic disposition. This study aims to develop an approach to determine unbound intracellular concentrations ($C_{u,cell}$) of ritonavir. Ritonavir was selected as a model drug as incubations with high ritonavir concentrations inhibited all saturable processes involved in ritonavir disposition including metabolism and transporter-mediated membrane passage. Following this incubation, hepatocytes were re-equilibrated in fresh protein-containing medium before determination of extracellular unbound ritonavir concentrations. In the absence of metabolism and transport, unbound intracellular and unbound extracellular concentrations were identical. In parallel, total intracellular ritonavir concentrations (C_{cell}) were determined, enabling the calculation of intracellular free fractions ($f_{u,cell}$). Additionally, C_{cell} was determined after exposing hepatocytes to a therapeutically relevant concentration (0.5 μM). Multiplication of this concentration with $f_{u,cell}$ resulted in $C_{u,cell}$. Finally, $Kp_{u,u}$ (intracellular unbound drug accumulation ratio) was calculated. Exposure of rat and human hepatocytes to 0.5 μM ritonavir resulted in $C_{u,cell}$ of 12 ± 1 and 8 ± 1 nM. Corresponding $Kp_{u,u}$ values were 2.7 ± 0.5 and 1.4 ± 0.2. We present an *in vitro* method to determine $C_{u,cell}$ of ritonavir in intact hepatocytes. $C_{u,cell}$ obtained at clinically relevant extracellular concentrations are in accordance with concentrations known to inhibit cytochrome P450 and are achieved because of ritonavir accumulation in hepatocytes. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2378–2387, 2015

Keywords: *in vitro* models; hepatocytes; membrane transport; CYP enzymes; passive diffusion/transport

INTRODUCTION

Knowledge regarding intrahepatic drug concentrations is essential to accurately predict efficacy, toxicity, and drug–drug interactions (DDI). Moreover, the unbound intracellular drug concentration ($C_{u,cell}$) in hepatocytes is particularly important as this is considered to be the relevant concentration interacting with intracellular processes.[1] For example, the pharmacological effect of drugs (such as statins) that target proteins residing inside hepatocytes is directly dependent on $C_{u,cell}$.[2] These intracellular concentrations (C_{cell}) should also be considered for drugs exhibiting hepatotoxicity. Troglitazone, for instance, inhibits the bile salt export pump (BSEP) and therefore the efflux of bile acids across the canalicular membrane. This results in an intrahepatic accumulation of bile acids causing drug-induced liver injury. Therefore, the $C_{u,cell}$ of BSEP inhibitors is essential for the prediction of hepatotoxicity.[3] Furthermore, clearance of many drugs is mediated by drug transporters and metabolizing enzymes in the liver. Additionally, many compounds inhibit or induce these proteins. Consequently, clearance predictions as well as the effect of potential perpetrators causing DDI can only be correctly predicted with accurate knowledge of $C_{u,cell}$.[1] For example, Pfeifer et al.[4] recently observed that the presence of ritonavir, an inhibitor of multidrug resistance-associated protein (MRP) 2, did not affect biliary excretion of ^{99m}Tc-mebrofenin, a MRP2 substrate, in sandwich cultures. However, intracellular ritonavir concentrations exceeded the IC50 value against MRP2-mediated transport of ^{99m}Tc-mebrofenin determined in membrane vesicles. On the basis of the binding of ritonavir to lysate of human hepatocytes, the authors suggest that the $C_{u,cell}$ of ritonavir was substantially lower than the concentration necessary to effectively inhibit MRP2.[4] Consequently, knowledge of $C_{u,cell}$ in hepatocytes is crucial in drug development to improve predictions of hepatic drug disposition.

Traditionally, the free drug hypothesis assumes the steady-state unbound plasma or blood concentration to be a surrogate marker for the $C_{u,cell}$.[5] In case the unbound concentration in the intracellular compartment is identical to the unbound concentration in the extracellular compartment, the intracellular unbound drug accumulation ratio ($Kp_{u,u}$) is 1. However, concentrations of free drug molecules at the target or metabolizing enzymes often deviate from the concentration in the systemic circulation ($Kp_{u,u} \neq 1$).[6,7]

Currently, methods that directly measure $C_{u,cell}$ are not available. However, quantitative microscopic chemical imaging approaches have been developed to determine compound accumulation in specific organs including the liver. Subsequently, mechanistic modeling is required to acquire $C_{u,cell}$.[1] Intrahepatic unbound fractions of drugs have also been estimated using equilibrium dialysis performed with hepatocytes incubated with metabolic inhibitors.[8] However, the presence of inhibitors might interfere with the intracellular binding kinetics of the compound of interest. Alternatively, equilibrium dialysis has been performed with cell or tissue homogenate[7,9] on the assumption that the destruction of the intact cellular context would not influence intrahepatic binding processes. However,

Abbreviations used: ABT, 1-aminobenzotriazole; BSEP, bile salt export pump; C_{cell}, intracellular concentration; C_{medium}, medium concentration; $C_{u,cell}$, unbound intracellular concentration; $C_{u,medium}$, unbound concentration in the medium; CV, coefficient of variation; CYP, cytochrome P450; DDI, drug–drug interaction(s); DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; f_u, unbound fraction; $f_{u,cell}$, unbound intracellular fraction; $f_{u,FBS}$, unbound fraction in FBS; $f_{u,medium}$, unbound fraction in medium; $f_{u,plasma}$, unbound fraction in human plasma; Kp, intracellular drug accumulation ratio; $Kp_{u,u}$, intracellular unbound drug accumulation ratio; Mrp/MRP, multidrug resistance-associated protein; Oatp/OATP, organic anion transporting polypeptide; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; RTV, ritonavir.

Correspondence to: P. Annaert (Telephone: +32-16-3-30303; Fax: +32-16-3-30305; E-mail: pieter.annaert@pharm.kuleuven.be)

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because of the lack of direct measurements, assumptions made under artifactual conditions are difficult to validate. As a result, refinement of existing methods and further methodological innovation is necessary to expand knowledge and improve predictions regarding drug disposition in the liver.[1]

Ritonavir is a widely used pharmacokinetic booster that targets cytochrome P450 (CYP) enzymes inside hepatocytes. The potent inhibition of CYP-mediated metabolism results in an increase in the bioavailability of concomitantly administered protease inhibitors used in antiretroviral therapy. Apart from an inhibitory effect, ritonavir is a substrate of CYP3A and CYP2D6, although the contribution of the latter to its hepatic clearance is only minor.[10] Moreover, the booster has been reported to be a substrate as well as inhibitor of human organic anion-transporting polypeptide (OATP) 1B1 and 1B3,[11,12] rat and human Mrp2/MRP2,[13,14] and P-glycoprotein (P-gp).[15] Involvement of active uptake processes, possibly Oatp-mediated, have also been observed in rat hepatocytes.[12]

In the present study, the objective was to develop and apply a novel *in vitro* method to determine unbound intrahepatic concentrations of ritonavir in intact hepatocytes. Therefore, metabolism and membrane transport have to be inhibited. When only passive diffusion remains, intracellular and extracellular unbound concentrations at equilibrium can be considered identical. Ritonavir was specifically selected as a model compound as it extensively inhibits its own metabolic and transport processes. Hence, no additional compounds are required that possibly interfere with intracellular binding of ritonavir.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM), William's E medium, phosphate-buffered saline (PBS), penicillin–streptomycin mixture (containing 10,000 IU/mL potassium penicillin and 10,000 μg/mL of streptomycin sulfate), L-glutamine, and fetal bovine serum (FBS) were obtained from Westburg (Leusden, The Netherlands). Ammonium acetate, acetic acid, methanol, and acetonitrile were purchased from VWR International (Leuven, Belgium). Ritonavir was provided by the National Institute of Health AIDS Research and Reference Reagent Program (Germantown, Maryland). All other chemicals were purchased from Sigma–Aldrich (St Louis, Missouri). Human plasma was provided by Red Cross (Leuven, Belgium).

Hepatocyte Isolation

Hepatocytes were isolated from male Wistar rats (175–200 g) with a collagenase perfusion as described previously.[16] Approval for the experiments was granted by the Institutional Ethical Committee for Animal Experimentation of KU Leuven. After isolation, cells were centrifuged (50*g*) for 3 min at 4°C and the pellet was resuspended in FBS. Viability was determined using the trypan blue exclusion method and was always higher than 85%.

Thawing Human Hepatocytes

A pool of human hepatocytes (lot number P2209A; 23 donors) was provided by Kaly-Cell (Plobsheim, France). Cryovials were removed from liquid nitrogen and immersed into a 37°C water bath. Immediately after all ice crystals were melted, hepatocytes were transferred to a tube containing 25 mL thawing medium [DMEM supplemented with 10% FBS (v/v), 2 mM L-glutamine, 1 μM dexamethasone, 4 μg/mL insulin, 100 IU/mL penicillin, 100 μg/mL streptomycin] and 16 mL isotonic Percoll (90% Percoll®, 10% 10× PBS) at 37°C. The volume was adjusted to 50 mL with thawing medium (37°C) and centrifuged at 168*g* for 20 min at room temperature. Subsequently, supernatant was removed, the pellet was resuspended in 20 mL thawing medium, and the suspension was centrifuged at 100*g* for 5 min at room temperature. Supernatant was removed and cells were resuspended in human plasma. Cell viability was determined using the Trypan blue exclusion method.

Equilibrium Dialysis

The unbound fraction (f_u) of ritonavir in FBS ($f_{u,FBS}$) or human plasma ($f_{u,plasma}$) was determined with equilibrium dialysis in the HTD96b from HTDialysis, LLC (Gales Ferry, Connecticut). FBS or human plasma containing ritonavir was added to the donor compartment; PBS was added to the acceptor compartment. The device was placed on a rotating incubator (KS 4000i Control incubator shaker; Staufen, Germany) for 6 h at 37°C to reach equilibrium, before samples ($n = 4$) of both compartments were taken. The f_u was determined:

$$f_u = \frac{C_{acceptor}}{C_{donor}} \quad (1)$$

Incubations in the Absence of Transporter and Enzyme Activity

Suspended rat or human hepatocytes (5×10^6 cells/mL) in FBS (rat) or human plasma (human) were loaded with 20 or 40 μM ritonavir in 50 mL tubes at 37°C for 10 min. Subsequently, hepatocytes were centrifuged at 50*g* for 3 min (rat) or 100*g* for 5 min (human) at 4°C and resuspended in fresh FBS (rat) or human plasma (human) at 4°C or 37°C to initiate the equilibrating phase (Fig. 1). The aim of this procedure was to inhibit rat and human transporters and enzymes involved in ritonavir disposition: (1) Oatp/OATP,[11,12] (2) Mrp2/MRP2,[13,14] (3) P-gp,[15] and (4) CYP.[10]

To verify whether transporter inhibition was maintained in the equilibrating phase, rat hepatocytes were incubated at 37°C for 10 min, immediately after resuspension, in the absence or presence of transporter inhibitors: (1) MK-571 (10 or 30 μM), Mrp-inhibitor; (2) benzbromarone (10 μM), Mrp-inhibitor; (3) zosuquidar (1 μM), P-gp-inhibitor; or (4) rifampicine (25 μM), Oatp-inhibitor. Subsequently, cells were treated with an additional amount of ritonavir for 30 s (1 μM) to facilitate the detection of residual transporter activity. After the incubation, an oil-spin was performed to sample hepatocytes (Fig. 1a). Briefly, aliquots of 200 μL cell suspension were transferred in triplicate to ice-cold microcentrifuge tubes (1.5 mL), containing 700 μL of an oil layer (density 1.015; a mixture of silicone oil and paraffin oil) above 300 μL of 8% NaCl solution. Tubes were centrifuged at 20,817*g* for 3 min in a tabletop centrifuge (Eppendorf 5415 C, Hamburg, Germany) and frozen in dry ice. After freezing, the tube bottoms were cut and the cell lysate was solubilized in 300-μL acetonitrile–water (70/30) containing the internal standard lopinavir (0.5 μM). The equilibrating phase was also executed at 4°C to determine nonsaturable distribution.

To verify inhibition of metabolic enzymes in the equilibrating phase, rat hepatocytes were incubated at 37°C for 10 min in