Evaluating the potential for lysosomal trapping in immortalized human hepatocytes (Fa2N-4 cells)

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INTRODUCTION

Sequestration of high concentrations of lipophilic amines (a.k.a. cationic amphiphilic drugs or CADs) in the acidic (pH 4-5) environment of lysosomes contributes to their presystemic clearance (by lysosomal trapping in liver and lung), their large volume of distribution (Vd) and their propensity to cause phospholipidosis (Hanumegowda *et al*, 2010; Daniel and Wojcikowski, 1999). In a related study with Lysotracker Red, a fluorescent probe that, by virtue of being a lipophilic amine, accumulates in lysosomes, we demonstrated that Fa2N-4 cells (immortalized human hepatocytes) contain functional lysosomes, similar to those observed in non-transformed human hepatocytes (Kazmi *et al*, 2011). However, Fa2N-4 cells do not retain uptake transporter activity (Harisparsad *et al*, 2008). In the present study, we measured the accumulation of two lysosomotropic probe drugs, namely propranolol and imipramine (lipophilic amines) and an acidic drug, atorvastatin (OATP substrate), in cultured Fa2N-4 cells in the presence and absence of lysosomotropic and non-lysosomotropic drugs (Nadanaciva *et al*, 2011). The aim of this study was to demonstrate that the lipophilic amines, propranolol and imipramine will undergo uptake into plated immortalized Fa2N-4 cells, whereas the OATP substrate, atorvastatin will not be taken up, and to ascertain whether the uptake of propranolol and imipramine would be inhibited by other lysosomotropics and the ionophores, nigericin and monensin.

METHODS

Chemicals: Amitriptyline HCl, astemizole, dextromethorphan, diclofenac, DMSO, imipramine, MFE Support Medium F (MFE) and propranolol were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium chloride was purchased from Aldrich (St. Louis, MO). Atorvastatin and the deuterated internal standard, d_5-atorvastatin were purchased from Toronto Research Chemicals (North York, ON, Canada). The deuterated internal standard, d_7-propranolol was purchased from CDN isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA). Cryopreserved immortalized hepatocytes (Fa2N-4 cells, lot 1010278) were prepared by XenoTech (Lenexa, KS).

Quantitative assessment of uptake by passive diffusion and lysosomal trapping:
Cryopreserved immortalized Fa2N-4 cells were cultured in flasks (T-150, Corning catalog number 430825) in MFE medium (support medium did not contain serum). The support medium was replaced every other day and the cells transferred when confluent (about twice a week). Before experiments, Fa2N-4 cells were plated on collagen-coated 96-well plates (Nunclon Delta SI) in MFE medium (10% serum) at a concentration of 50,000 cells/well. Cells were maintained at 37°C with 95% humidity and 5% CO_2. Following the cell attachment period (3-20 hours), media was replaced with fresh media that did not contain serum and experiments were performed 36-48 hours post-plating. Substrates (1 µM) were incubated with plated Fa2N-4 cells in MFE media (without serum) at 37±1°C in the presence and absence of additional CADs, compounds thought to inhibit uptake transporters, ammonium chloride (disrupts the proton gradient of the lysosome) and nigericin and monensin (ionophores, which disrupt lysososomal function by increasing the internal pH, leading to osmotic lysis of the lysosome (Arai *et al*, 2002)). The final incubation volume was 100 µL. Before the incubations were started, the cells were washed once with fresh MFE media (without serum). Cells and the substrate solution were pre-incubated separately and incubations were initiated by the addition of the substrate solution with or without inhibitor solution to the cells. Incubations were terminated by the addition of ice-cold PBS (100 µL). The cells were then washed two times with ice-cold PBS (100 µL) and were subsequently lysed with acetonitrile containing internal standard (150 µL). An aliquot (100 µL) of the cell lysate was transferred to a 96 well deep well analytical plate. Water was added to the samples to bring the total volume of each well to 200 µL for analysis. The 96-well analytical plate was then vortexed and centrifuged at 920 RCF for 10 min at 10°C to pellet residual protein. The amount of substrate trapped in the cells was monitored by LC/MS/MS detection (API 2000 for propranolol and imipramine, API 3000 for Atorvastatin).

RESULTS

Figure 1 shows that both propranolol and imipramine (CADs) undergo uptake in Fa2N-4 cells and that the uptake was inhibited by 50 mM NH_4Cl (which neutralizes the pH of the lysosome), whereas little or no atorvastatin uptake was observed after 5 min, and NH_4Cl had no effect on atorvastatin uptake.

Figure 1

Uptake of propranolol, imipramine and atorvastatin after 5 min and the effect of NH_4Cl on uptake

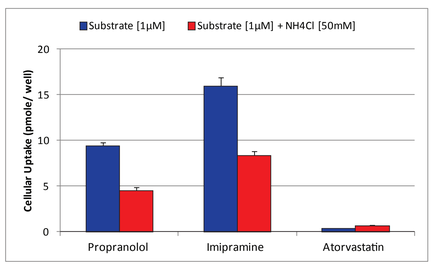


Figure 2 shows that the uptake of propranolol and imipramine was linear up to ~10 min and appeared to plateau at ~10 min (propranolol) or 20 min (imipramine). In the presence of NH_4Cl, the trapping of propranolol and imipramine was reduced by about 60 and 45%, respectively (at 10 min). The level of uptake in the presence of NH_4Cl may be possibly due to non-specific binding to intra- and extracellular components of the Fa2N-4 cells.

Figure 2

Time-dependent uptake of propranolol and imipramine in Fa2N-4 cells and the affect of NH_4Cl on uptake

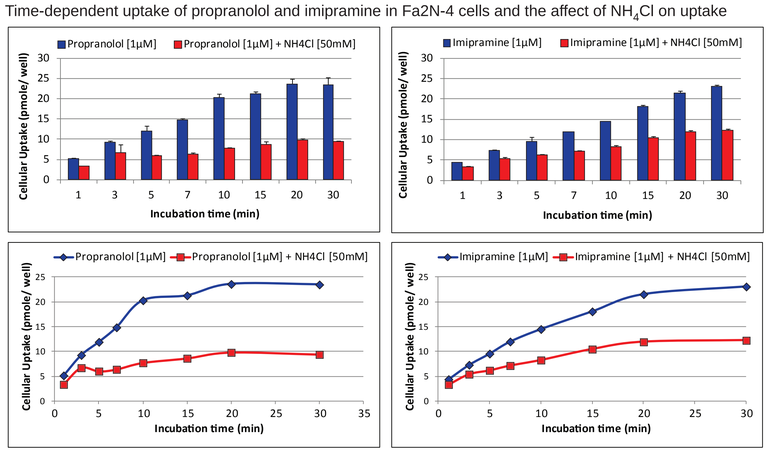


Figure 3 shows the effects of inhibitors of lysosomal trapping, namely NH_4Cl and chloroquine (a known lysosomotropic compound), on the uptake of propranolol when a) the substrate and inhibitor are co-incubated, b) the inhibitor is pre-incubated with the cells then co-incubated with the cells and c) a pre-incubation step only. Similar effects were seen from both pre-incubation followed by co-incubation and co-incubation only with both inhibitors. The inhibitory effects of NH_4Cl can be reversed if it is removed prior to incubation with propranolol, however, the inhibitory effects of chloroquine remain after it is removed from the incubation media, presumably because it is trapped in lysosomes and its inhibitory potential is not diminished with a short wash out period.

Figure 3

Effect of incubation techniques on inhibition of propranolol uptake

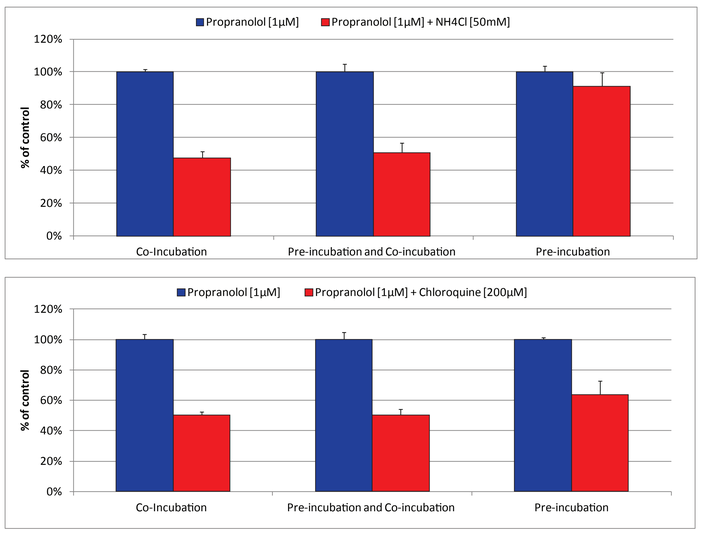


Figure 4 shows the inhibition effects of a) NH_4Cl, b) nigericin + monensin and c) NH_4Cl + nigericin + monensin on the uptake of propranolol and imipramine into the Fa2N-4 cells. Adding multiple inhibitors does not increase the level of inhibition, which further suggests that uptake of 4-5 pmole/well of propranolol or 6 pmole/well of imipramine is likely due to non-specific uptake or binding.

Figure 4

Inhibition of propranolol and imipramine: additive effects with multiple inhibitors of lysosomal trapping

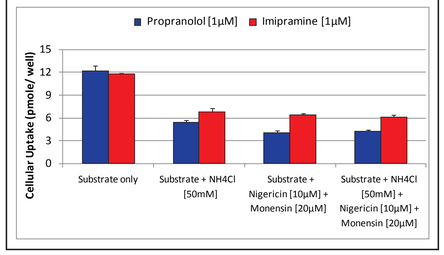
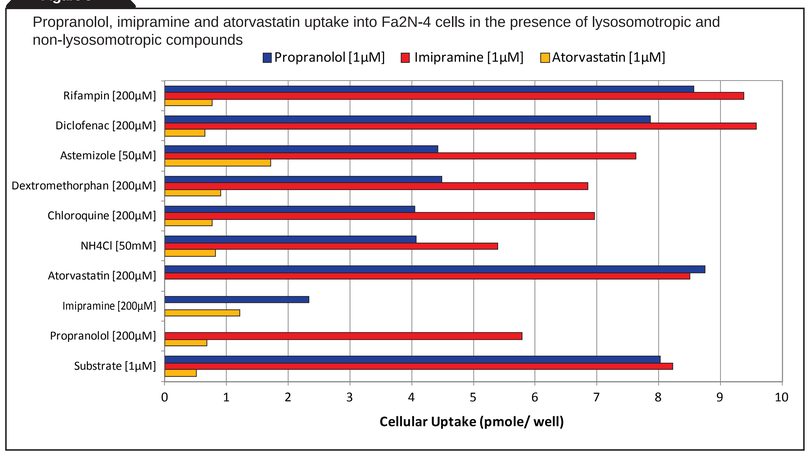


Figure 5 shows inhibition of uptake of propranolol, imipramine and atorvastatin by a) lysosomotropic compounds, b) non-lysosomotropic compounds and c) NH_4Cl. The uptake of both propranolol and imipramine is inhibited by NH_4Cl which neutralizes the pH of the lysosome as well as the lysosomotropic compounds, astemizole, dextromethorphan, chloroquine and imipramine or propranolol, respectively, but not by diclofenac (acid), rifampin (OATP inhibitor) or atorvastatin (acid and OATP substrate). Compared to imipramine and propranolol, very little atorvastatin was taken up into the Fa2N-4 cells. The addition of inhibitors thought to inhibit lysosomal trapping have little to no effect on atorvastatin uptake.

Figure 5

Propranolol, imipramine and atorvastatin uptake into Fa2N-4 cells in the presence of lysosomotropic and non-lysosomotropic compounds



CONCLUSIONS

- Lipophilic amines are taken up into the lysosomes of Fa2N-4 cells and their uptake is inhibited by lysosomotropic drugs and ionophores. Fa2N-4 cells may be a useful and practical alternative to cultured human hepatocytes to determine whether drug candidates are subject to lysosomal trapping.
- The OATP substrate, atorvastatin was not taken up into the lysosomes of the Fa2N-4 cells, and the addition of lyososomtropic drugs had no effect on atorvastatin uptake, further indicating that Fa2N-4 cells do not retain uptake transporter activity.

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