

# Mouse Bsep ATPase assay – a non-radioactive tool for assessment of the cholestatic potential of drugs



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## INTRODUCTION

Bile salts are the major organic solutes in bile obtained by enzymatic catabolism of cholesterol synthesized within hepatocytes and transported mainly by an ABC efflux transporter the Bile Salt Export Pump (BSEP, ABCB11). The gene encoding BSEP, ABCB11 is mutated in what is known as BSEP deficiency or progressive familial intrahepatic cholestasis type 2 (PFIC2)<sup>1</sup>. In humans there is no compensatory mechanism for the loss of this transporter<sup>2</sup>. Drug-induced cholestasis is a frequent problem in clinical medicine hence there is a real need for reliable, validated screening tools suited to test drug candidates for BSEP interaction potential during drug development process.

In this study the mouse ortholog of the human BSEP transporter was expressed in baculovirus infected insect cell (Sf9) system in order to study the effect of membrane cholesterol content on the transport function. Physiologically the bile salt export pump is expressed on the cholesterol-rich canalicular membrane of liver cells meanwhile the lipid composition of the used insect cell membranes significantly differ, the cholesterol content of Sf9 plasma membranes being 5- to 10-fold lower than that of plasma membrane<sup>3</sup>. This difference can markedly affect the ATPase and transport activity as we showed previously<sup>4</sup>. Cholesterol enrichment of mBsep-Sf9 vesicles resulted in a membrane product that is suitable for non-radioactive high throughput screening for bile salt export pump interactors using the ATPase assay in a good rank order correlation with human BSEP TC transport.

## MATERIALS AND METHODS

### Membrane preparation and cholesterol loading

The insect membrane vesicles were produced using Sf9 insect cells infected with recombinant baculoviruses encoding mBsep. Sf9 cells were cultured and infected with a recombinant baculovirus stock as described earlier<sup>5</sup>. For cholesterol loading of the membranes cells were collected and treated with Hank's balanced salt solution (HBBS) containing 1 mM cholesterol@RAMEB for 30 min at 37 °C then washed. Cholesterol loading of membranes is indicated as „HAM” (High Activity Membranes)

### Vesicular transport assay

Inside-out vesicles were incubated in the absence or presence of 4 mM ATP at 37 °C, 5 min reaction time. Test compounds in increasing concentrations were co-incubated with 2 μM tritiated TCDC. Vesicles were separated by rapid filtration technique and retained radioactivity was measured by liquid scintillation method. ATP dependent transport was calculated by subtracting the values obtained in the absence of ATP from those in the presence of ATP.

### ATPase assay

ATPase activity was measured in the presence and absence of sodium orthovanadate applying the protocol of the PREDEASY ATPase Kit (Solvo Biotechnology, Hungary) in presence of 100 μM TCDC concentration.

## RESULTS AND DISCUSSION

The effects of cholesterol loading on the transport kinetics of the mBsep transporter was investigated in mBsep-Sf9 inside-out vesicles.

➤ [<sup>3</sup>H]TCDC transport is time and concentration dependent with increased  $V_{max}$  values in HAM-Sf9 membrane vesicles (967 ± 8 versus 525 ± 5) with a slight shift in  $K_m$  values (Fig. 1/A)

➤ concentration dependent transport of TCDC in ATPase assay was also assessed with decreased basal (vanadate sensitive) activity and increased maximal stimulation in cholesterol uploaded membrane vesicles with similar  $EC_{50}$  values (16.5 μM) (Fig. 1/B)

➤ maximal TCDC induced vanadate sensitive ATPase was reached at about 2 mM ATP concentration (Fig. 2/A) and a best signal-to-background ratio at 8 μg membrane protein/well concentration. (Fig. 2/B)

➤ various cholestatic drug molecules effect on TCDC transport and TCDC stimulated ATPase activity were measured in vesicular transport (Fig. 3/B) and ATPase assays (Fig. 3/A) and compared to hBSEP mediated TC transport. A high rank order correlation of  $IC_{50}$  values can be observed in the three different assays (Table 1.).

➤ The mBsep-HAM-Sf9 ATPase is a high throughput, sensitive, non-radioactive assay designated to detect drug – Bsep interaction.

### REFERENCES

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### ACKNOWLEDGEMENT

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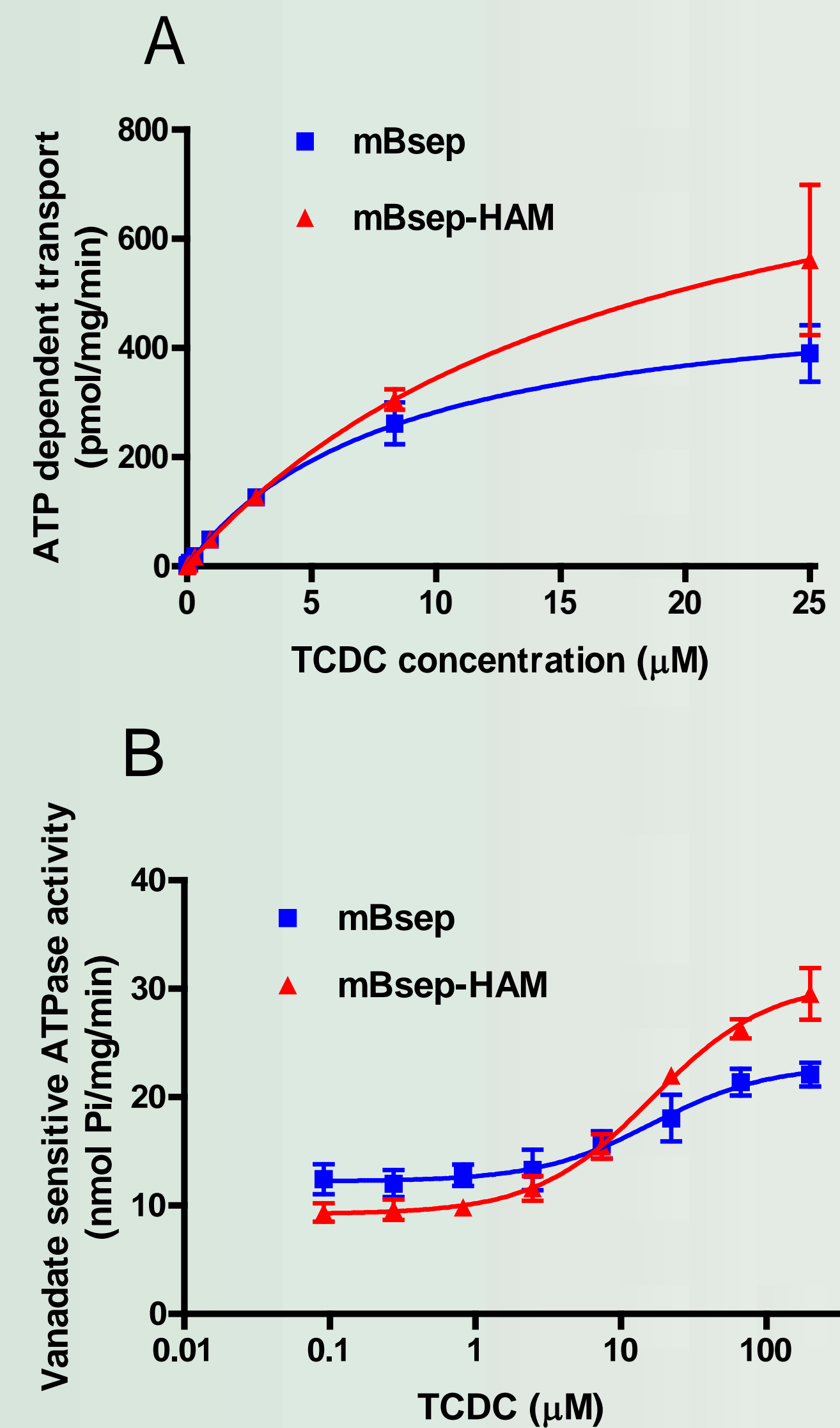


Figure 1: A) ATP dependent transport of [<sup>3</sup>H]TCDC in mBsep and mBsep-HAM-Sf9 inside-out vesicles. B) Stimulation of vanadate sensitive ATPase activity of mBsep and mBsep-HAM-Sf9 vesicles by TCDC.

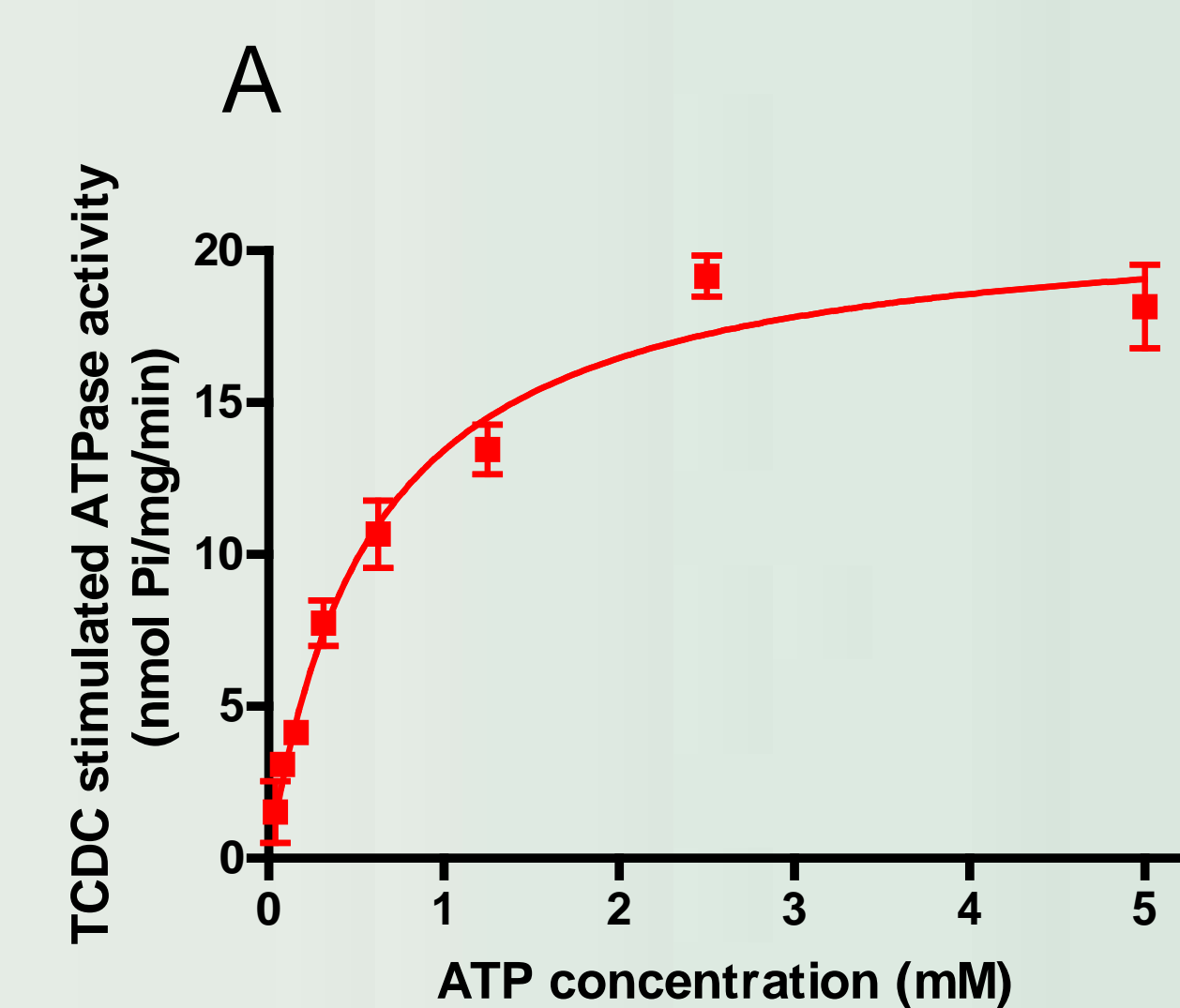


Figure 2: A) ATP concentration dependence of TCDC (100 μM) stimulated mBsep-HAM-Sf9 ATPase activity.

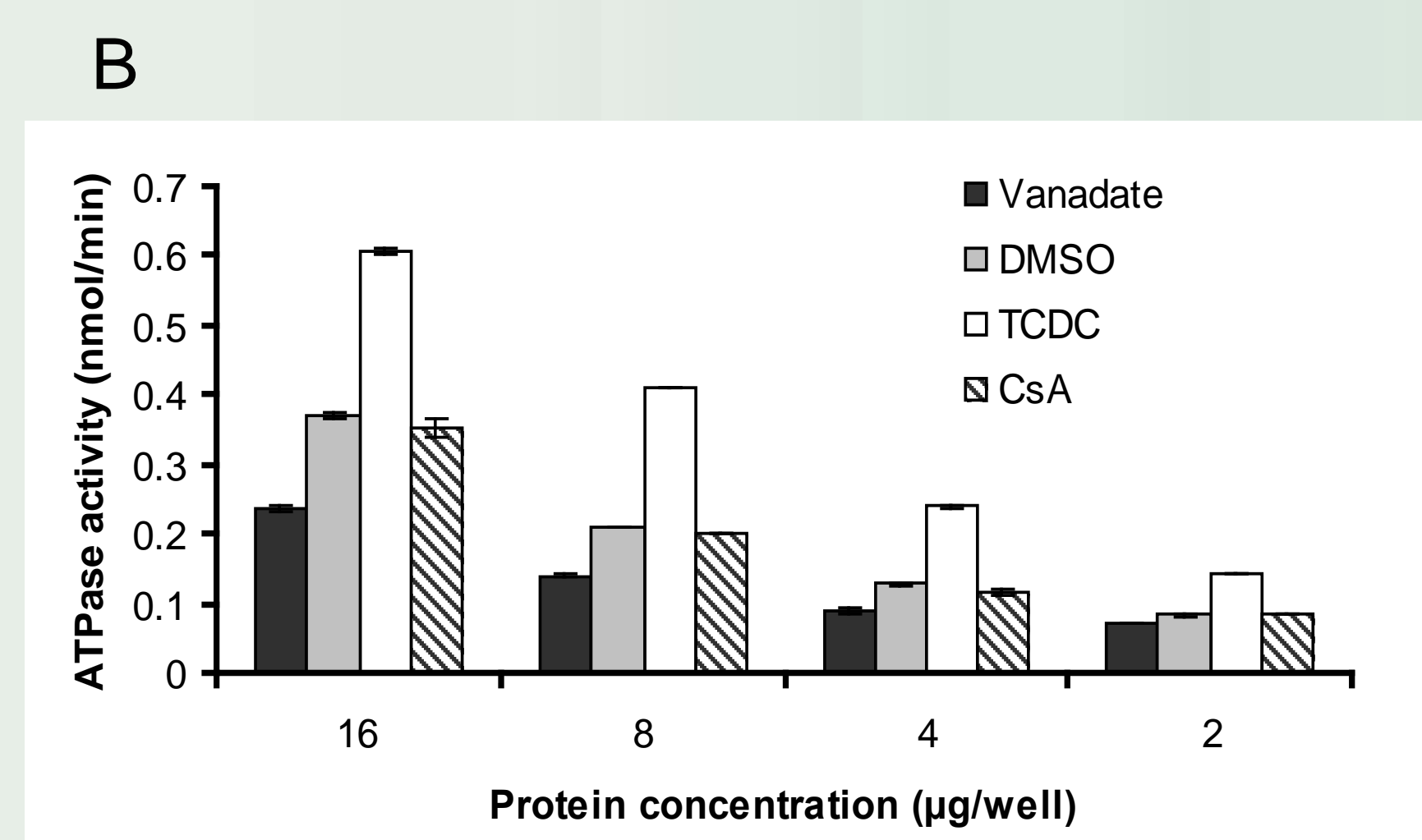


Figure 2: B) Vanadate sensitive ( ), background (DMSO), drug stimulated maximal ( ) and inhibited (Cyclosporin A) ATPase activities of mBsep-HAM-Sf9 membrane at different protein concentrations (μg/well). Vanadate insensitive activity was measured in the presence of 1.2 mM Na-orthovanadate, background in presence of 2% DMSO, maximal activity induced by 100 μM TCDC, inhibition by 100 μM Cyclosporin A.

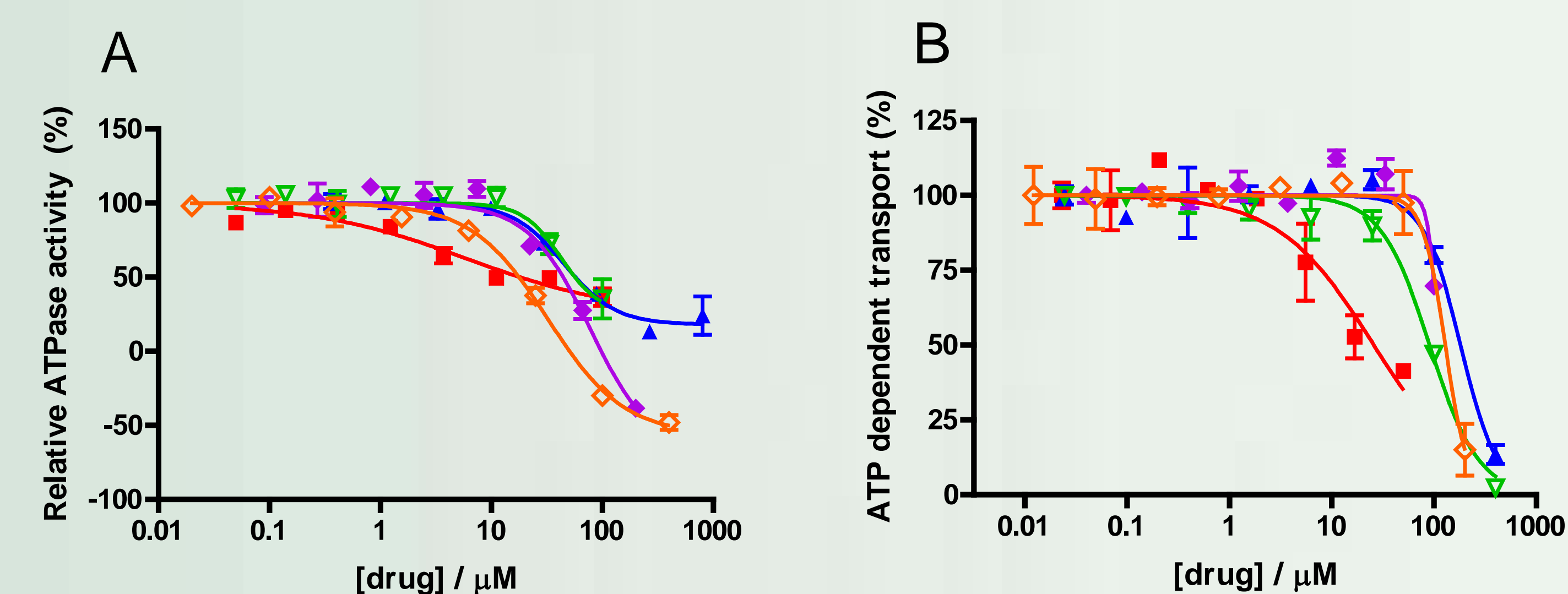


Figure 3: A) Inhibition of TCDC stimulated mBsep-HAM-Sf9 ATPase activity and B) inhibition of mBsep-mediated TCDC transport by the selected drug molecules using mBsep-HAM-Sf9 vesicles.

| Compound      | $IC_{50}$ values (μM)  |                          |                              |
|---------------|------------------------|--------------------------|------------------------------|
|               | TCDC stimulated ATPase | TCDC vesicular transport | hBSEP TC vesicular transport |
| Cyclosporin A | 3.5                    | 7.2                      | 20                           |
| Glibenclamide | 45                     | 120                      | 15                           |
| Rifampicin SV | 62                     | 105                      | 20                           |
| Ketoconazole  | 33                     | >100                     | 5.0                          |
| Troglitazone  | 33                     | 97                       | 8.0                          |
| Rifampicin    | >100                   | No interaction           | 50                           |

Table 1. Correlation between TCDC stimulated ATPase inhibition, TCDC transport inhibition data ( $IC_{50}$  values) and human BSEP transporter mediated TC transport inhibition.