

# New methods for the functional investigation of drug transporters

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## Background

In the past few years, the **role of OATPs (Organic Anion Transporting Polypeptides)** in drug disposition, drug clearance and drug-drug interactions (DDI) has been intensely investigated. ATP-binding cassette proteins (ABCs) have already been in the spotlight for a long time. Several members of the OATP and ABC families have partially overlapping substrate specificity which might be explained by the similar expression pattern of these proteins. *For instance, OATPs and ABCs work together in the hepatic clearance* (see Fig.I.). If the normal function of these proteins is interrupted by DDIs it might lead to serious consequences, such as toxicity. Therefore, predicting potential interactions with OATP- and ABC-transporters is of great scientific interest.

The major impediment to the investigation of OATP function is the limited availability of radioactively- or fluorescently-labeled substrates and OATP-specific inhibitors. Functional studies are also hindered by the limited number of cellular models with well-characterized transporter expression.

**In this study, our aims were:**

1. Development of comprehensive, fluorescence-based functional assays for the functional analysis of all 11 human OATPs.
2. Establishment of a fluorescence-based HTS (high-throughput) assay to screen for OATP-interacting molecules.

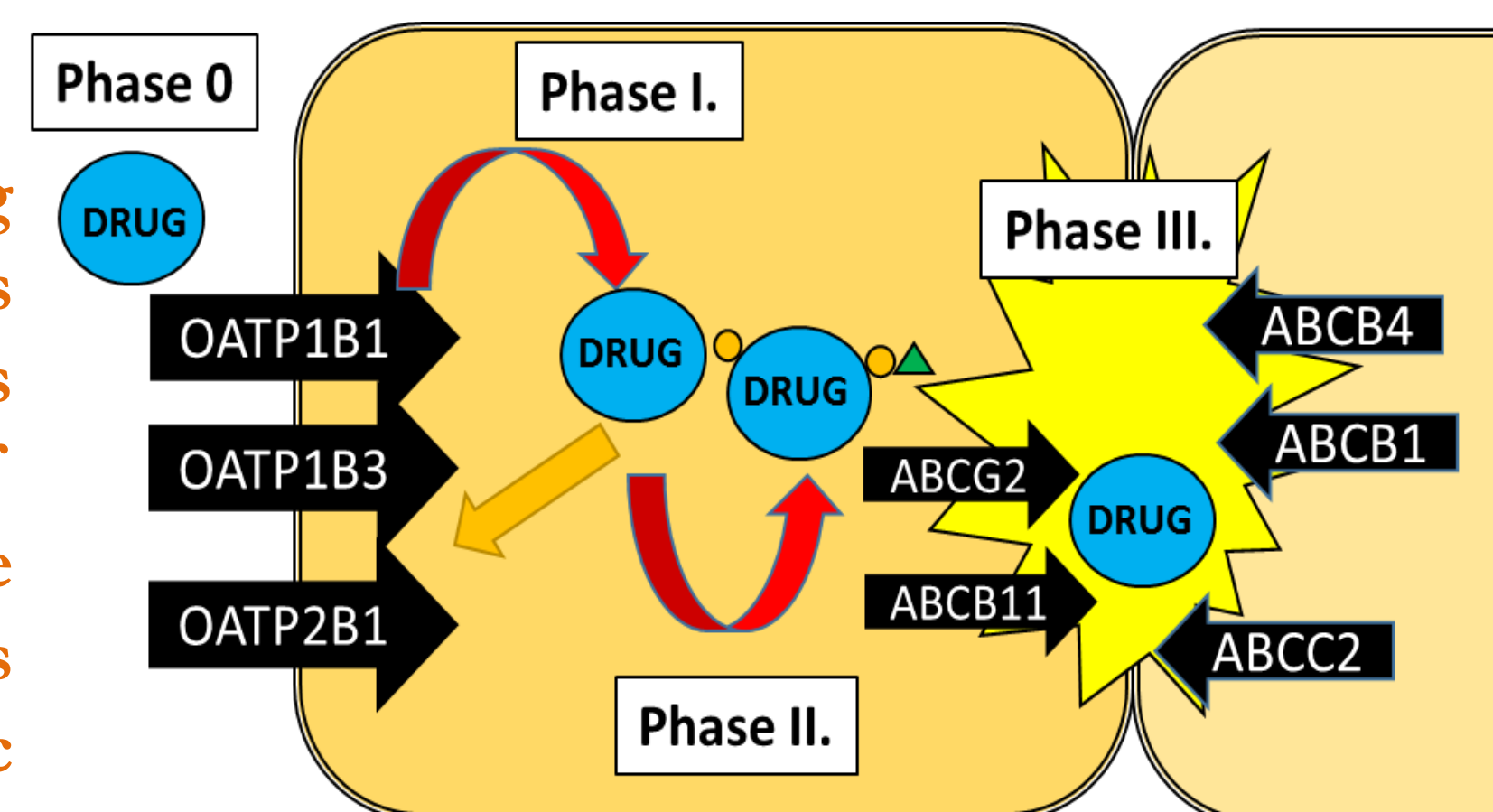


Fig. I.: Participation of OATPs (uptake) and ABC-transporters (efflux) in hepatic drug uptake.

## 1. Insect cell-based (*Spodoptera frugiperda*-Sf9) transport activity assay

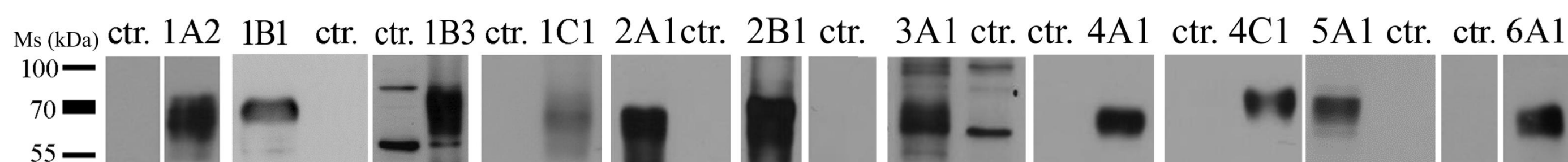
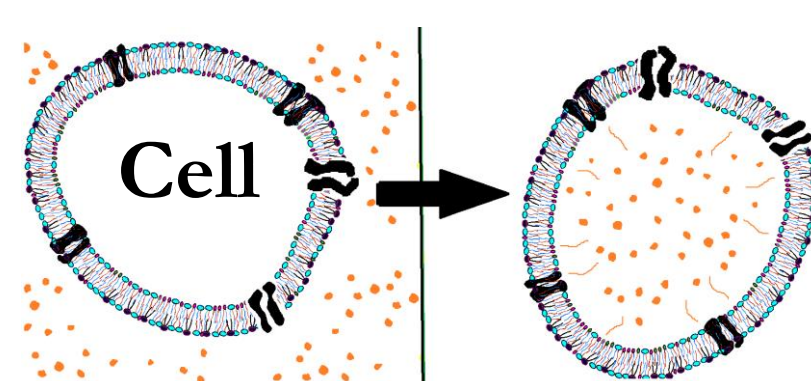


Fig. II.: After we confirmed the applicability of insect cells for the functional expression of OATP1B1 and 1B3 (data not shown), we have generated Sf9 cells overexpressing the full human OATP panel. Expression of the proteins was confirmed by Western blot using polyclonal antibodies.

### 1.a New fluorescence-based assay



### ►► Sodium-fluorescein as a pan-OATP substrate

Transient expression of all human OATPs (1A2, 1B1, 1B3, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1, 6A1) showed elevated sodium fluorescein (Na-Fluo) uptake as measured by flow cytometry. The assay can be used to analyze OATPs that are less characterized and to screen for OATP-interacting compounds.

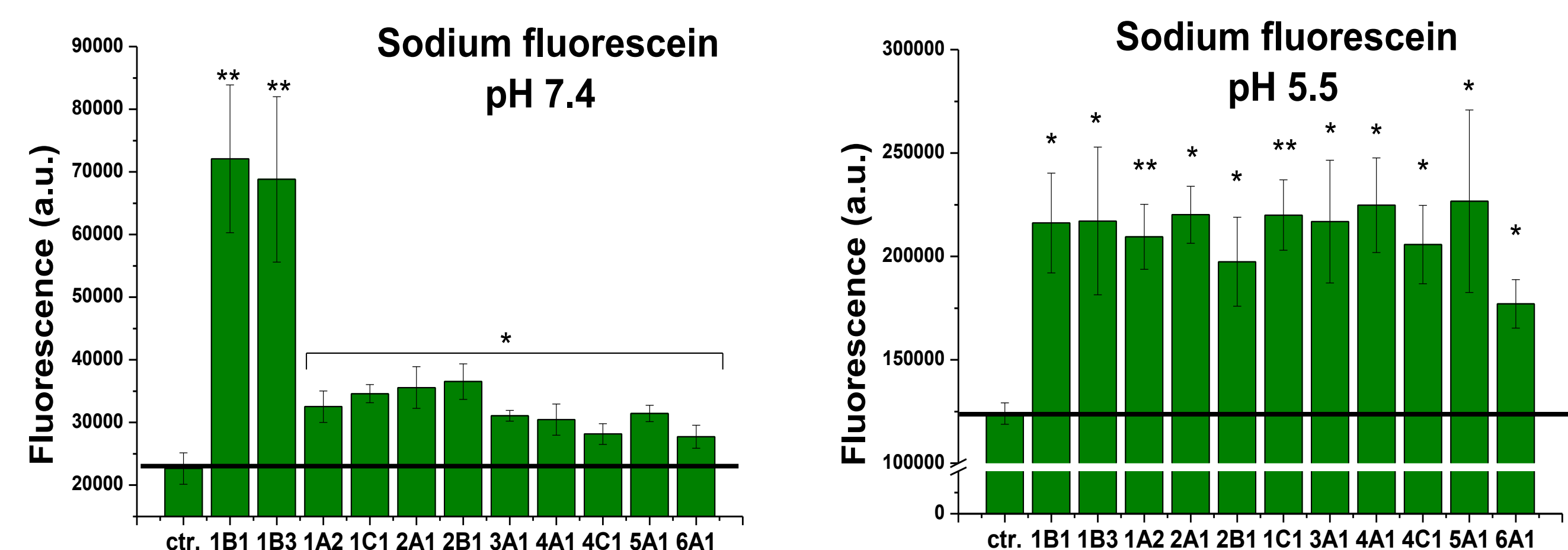


Fig. III.: Accumulation of 1  $\mu$ M Na-Fluo (a previously described OATP1B substrate) was measured in Sf9 cells overexpressing human OATPs for 10 minutes at pH 7.4 or pH 5.5. The acidic pH of the extracellular milieu can effect the structure and activity of several enzymes including OATPs. Therefore, we generated an inwardly directed  $H^+$  gradient to see if there will be an altered transport activity. We found that Na-Fluo transport was significantly enhanced, suggesting that the transport of Na-Fluo transport is triggered by a proton gradient. (Patik et al., Biochemical Pharmacology, 2015.)

### 1.b Newly discovered fluorescent OATP substrates

Fluorescent compounds are more convenient, safe and cost-efficient than isotope-based functional assays. We have proven that Na-Fluo is a suitable alternative to radiolabeled ligands; however, specific molecules with a higher signal-to noise ratio would be preferable. Using our newly established assay, we discovered new fluorescent substrates for OATP1B1, 1B3, 2B1, designated as NOS I-V (New OATP Substrate).

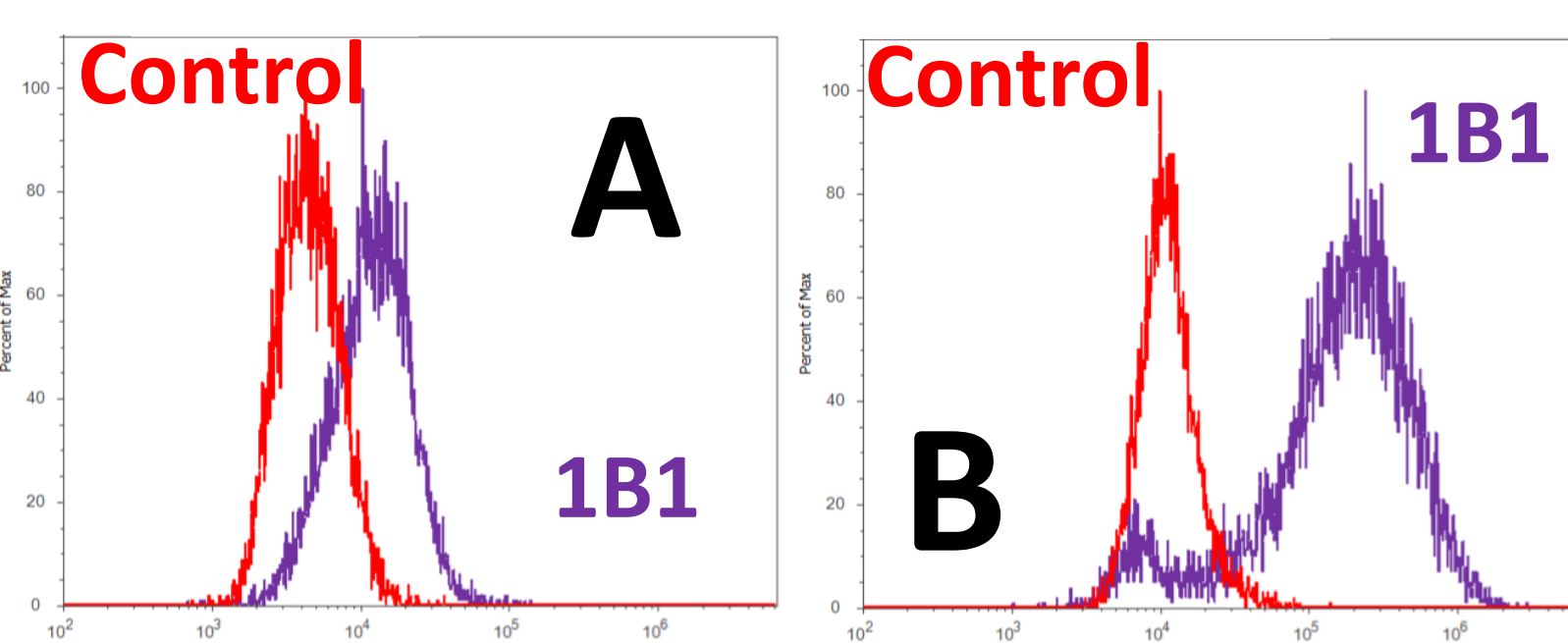


Fig. IV.: Representative histograms show the uptake of 1  $\mu$ M Na-Fluo (A) and 1 U NOS I (B) in Sf9 cells. Higher signal was observed for NOS I. As a negative control, cells were transfected with a baculovirus containing the cDNA of an unrelated protein.

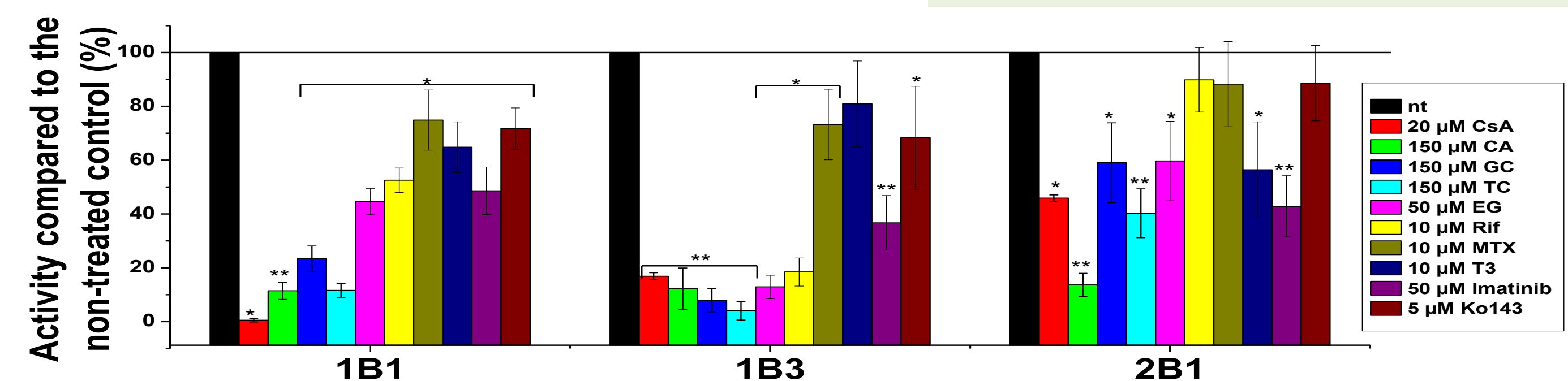


Fig. V.: We investigated several previously identified OATP1B1/1B3 interacting molecules to see their influence on NOS I uptake. We could detect a significant decrease in OATP transport activity for most of these compounds. We concluded that the observed activity was due to the overexpressed human OATP proteins. Cyclosporin A (CsA), cholic acid (CA), glycocholate (GC), taurocholate (TC), estradiol 17 $\beta$ -D-glucuronide (EG), rifampicin (Rif), methotrexate (MTX) and Imatinib in Uptake buffer adjusted to pH 5.5. The actual concentrations are indicated above. For statistical analysis, Student's test was used. \*:  $p < 0.05$ , \*\*:  $p < 0.01$

## 2. Mammalian cell based assay

To establish a 96-well plate assay that is suitable for the high-throughput screening of novel OATP-interacting molecules, we generated OATP-overexpressing A431 cells (strongly adherent epidermoid carcinoma cells).

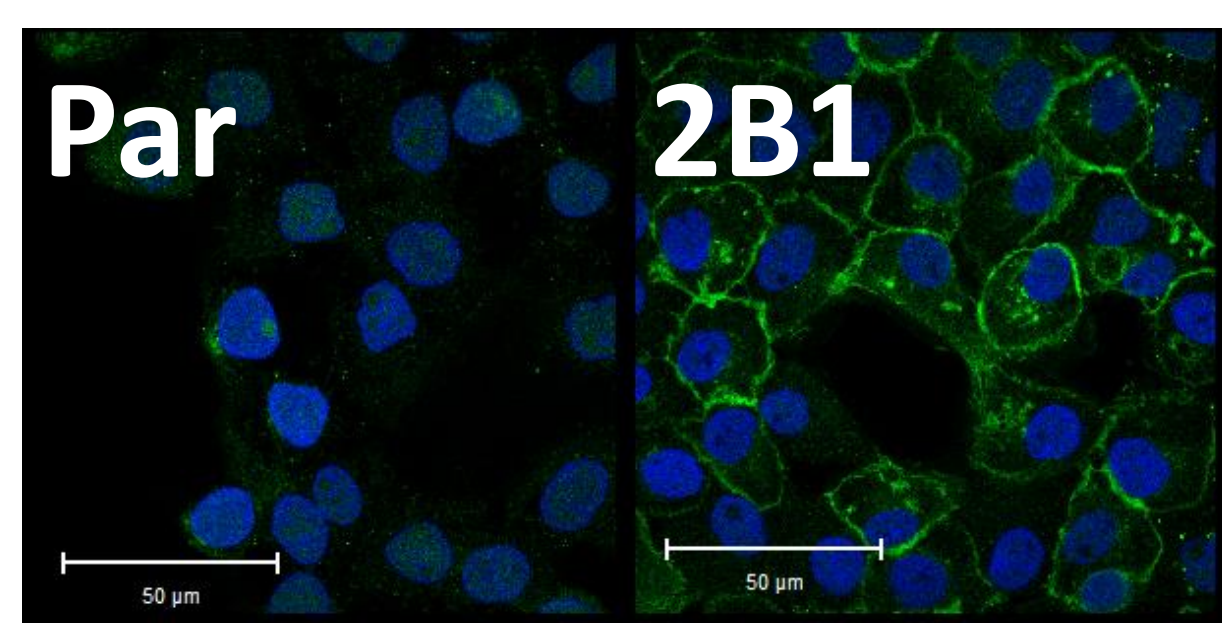


Fig. VI.: Immunofluorescent detection of OATP2B1 expressed in A431 cells.

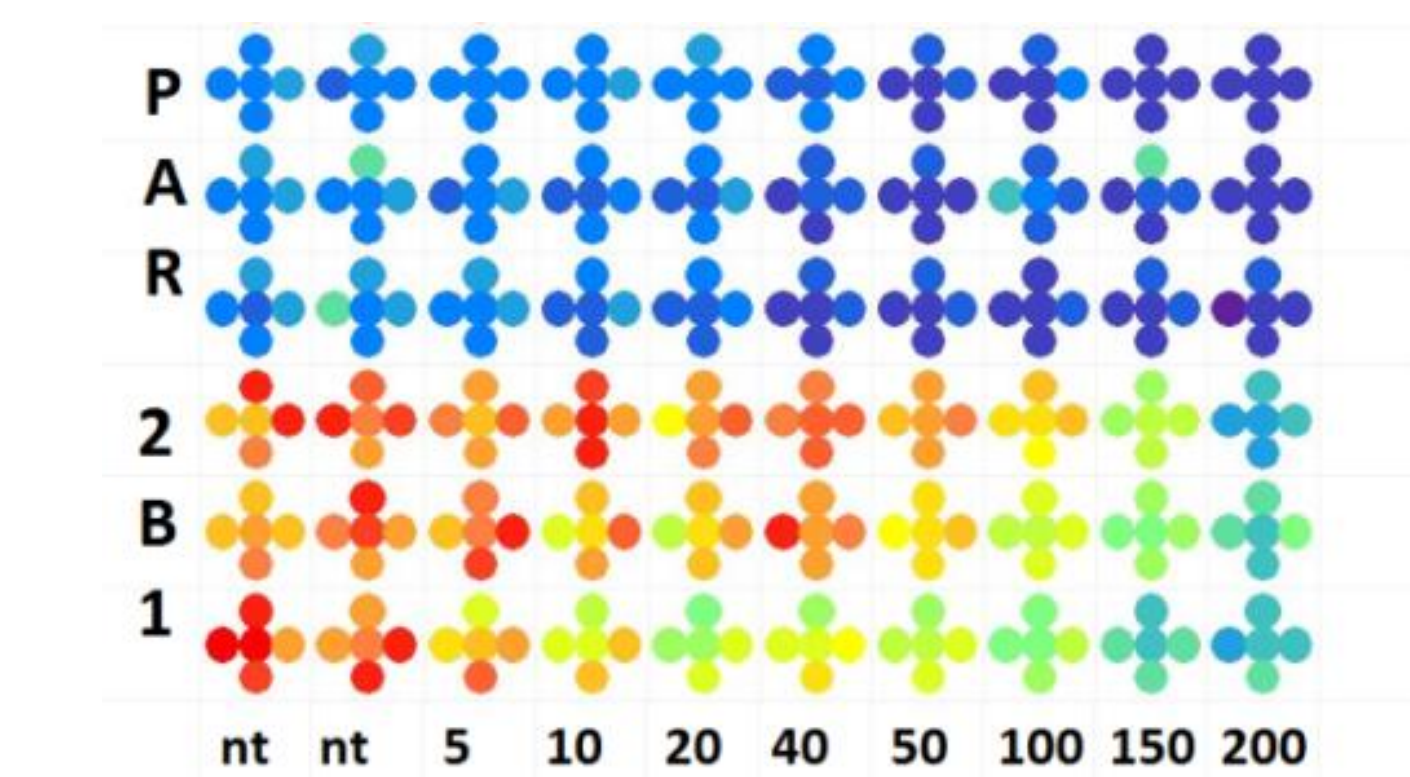


Fig. VII.: Concentration-dependent inhibition of NOS II uptake after 30 mins with a new OATP-interacting molecule. (Colored uptake intensity on 96-well plate)

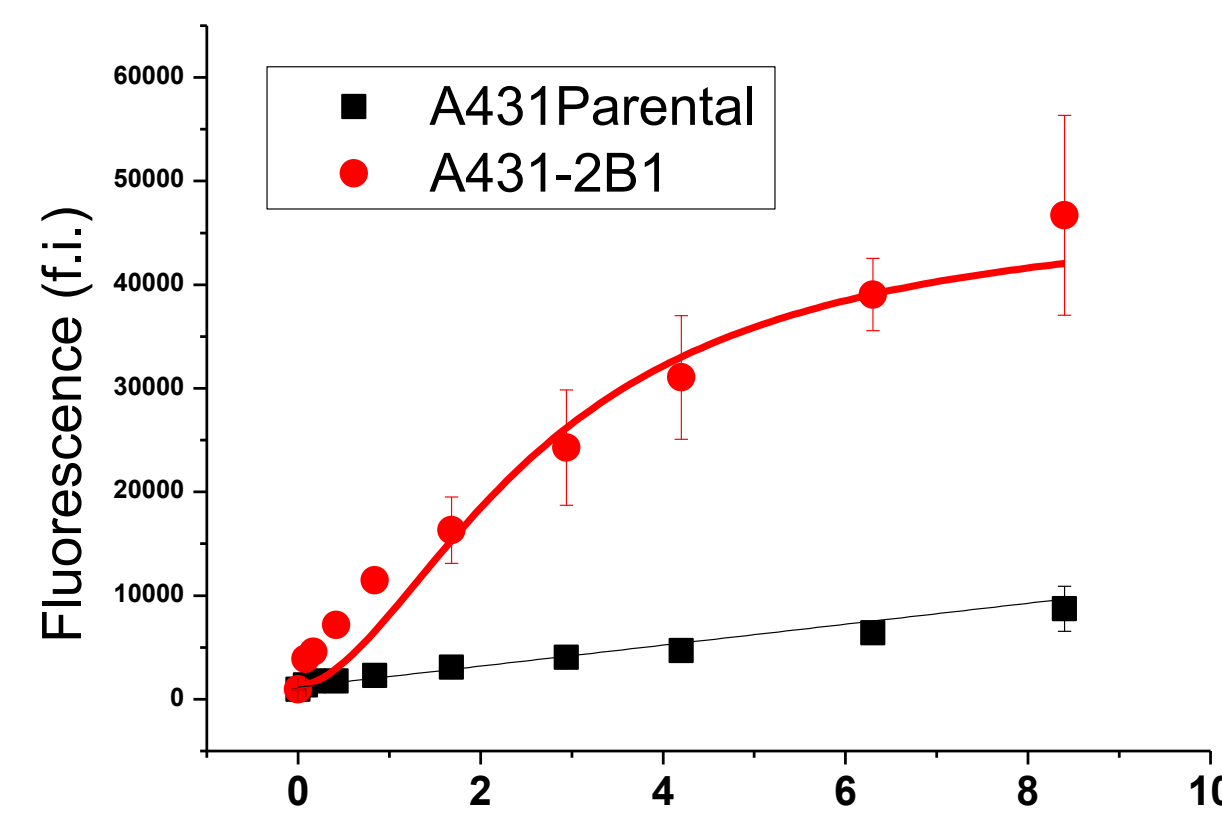


Fig. VIII.: Concentration-dependent uptake of NOS III in A431-OATP2B1 cells after 30 mins on 96-well plate.

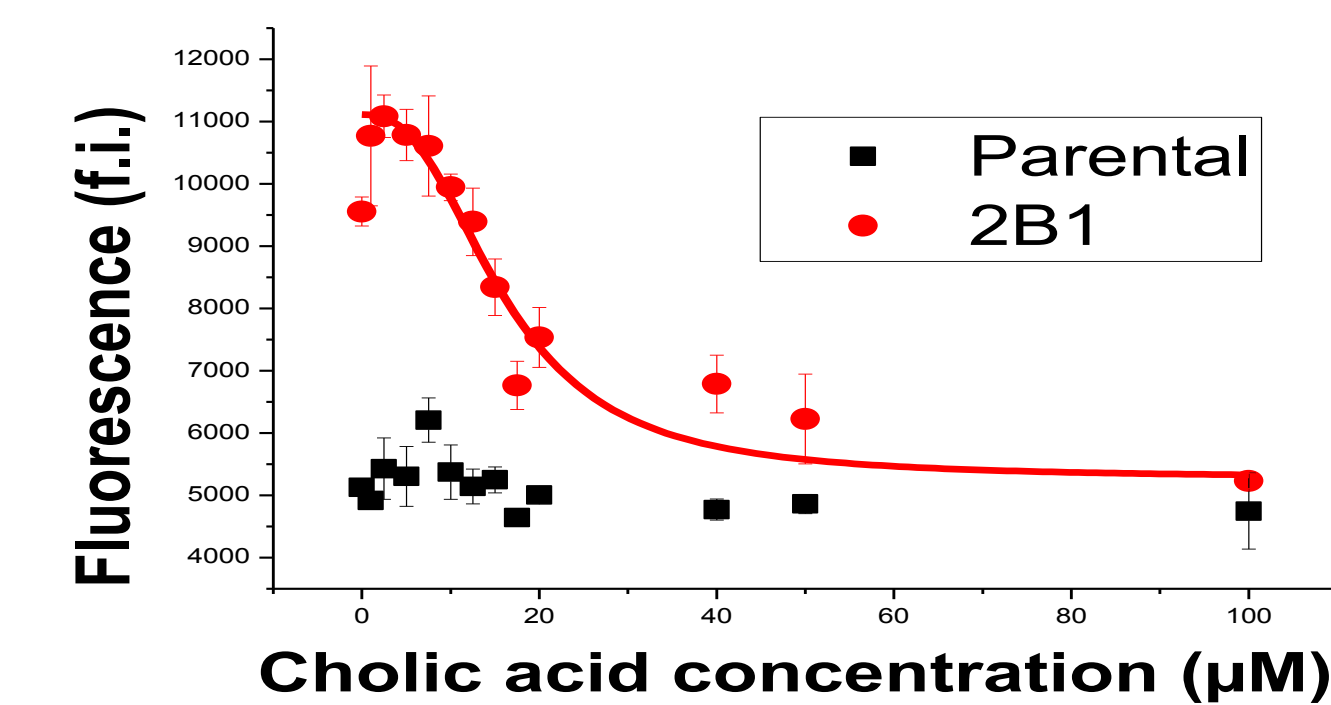


Fig. IX.: Concentration-dependent inhibition of NOS I uptake by cholic acid in A431-OATP2B1 cells after 30 mins on 96-well plate.

## 3. NOSs interact with ABCs

As previously described, ABCG2 and ABCC2 have overlapping substrate specificities with OATPs. Therefore, we tested if the new fluorescent compounds were transported by ABCs.

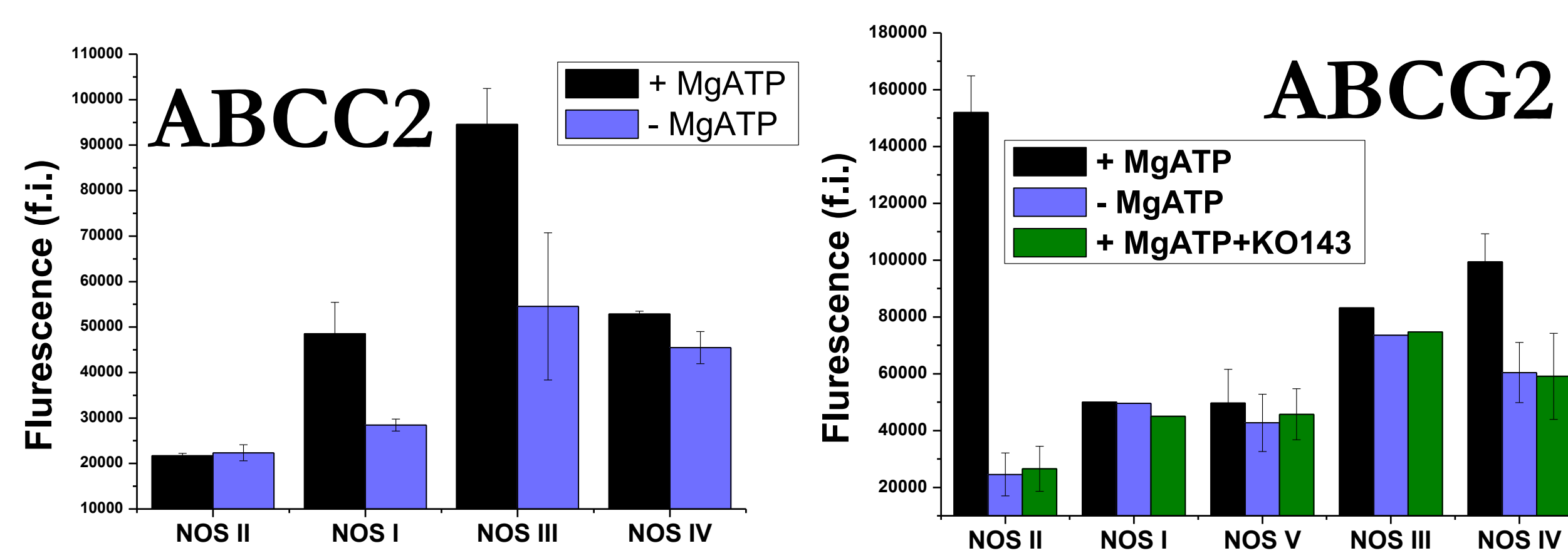


Fig. X.: NOSs uptake by ABCC2 (right) or ABCG2 (left) IOVs after 10 mins of incubation at 37°C.

## Results

- We demonstrate that sodium-fluorescein is transported by all human OATPs and this transport is increased when an acidic extracellular milieu is created.
- Sodium-fluorescein uptake can be modified by OATP-interacting compounds (Patik et al., 2015).
- The newly developed, Sf9-based method is the first fluorescence-based assay to investigate the entire human OATP family.
- We identified new fluorescent substrates for OATP1B1, 1B3, 2B1, ABCC2 and ABCG2.
- Using the NOSs, we developed a 96-well plate assay for OATPs, 1B1, 1B3 and 2B1.
- The newly developed cell-based assays are suitable for the functional characterization of human OATPs and allow the identification of new OATP substrates
- In addition, NOSs may be applied to develop novel fluorescent assays for ABCs.

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