Sensing transport: label-free in vitro assays as an atTRACTive alternative for solute carrier transporter drug discovery

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SUMMARY

Transport proteins are present in virtually all biological membranes of living organisms. These so-called 'transporters' are required for a cell to take up nutrients and excrete waste, which serves the purpose of ensuring that cell's growth and survival as well as facilitate its communication with neighboring cells. The largest class of transporters is the superfamily of solute carriers (SLCs), which in humans comprises over 450 members divided into 66 subfamilies, each with their own substrate specificity and transport mechanism. Due to their critical role in the proper functioning of cells, it may not be surprising that SLCs play a role in the development and progression of diseases. So far, SLCs have been the target of frequently prescribed drugs, such as neurotransmitter transporters (targets of antidepressants) and Na+/K+/Cl–-transporters (targets of loop diuretics). However, the majority of SLCs are poorly studied despite their tremendous potential as drug targets. Thus, there is a need for the development of new methods that can be used to study the function of SLCs as well as identify modulators (e.g., inhibitors). The advantages and limitations of current in vitro methods to study SLCs are summarized in Chapter 1. Moreover, the concept of cell-based label-free assays is introduced as an innovative approach to assess transport function in a setting that is closer to a cell's physiological environment. Throughout the five experimental chapters of this thesis, the impedance-based technology xCELLigence is used as a core platform for the development and application of novel functional assays for three types of SLC: dopamine (DAT), norepinephrine (NET) and glutamate (EAAT) transporters.

In humans, activation of G protein-coupled receptors (GPCRs) on the cell surface and other cellular membranes leads to cellular responses that control a wide variety of physiological processes. Some of the endogenous ligands that activate these GPCRs – such as neurotransmitters or signaling lipids – are transported from the extracellular space into the cell by SLCs. This regulation is necessary to prevent excessive GPCR activation, which is at the basis of drugs that inhibit such SLCs (i.e., antidepressants) and increase extracellular amounts of the endogenous ligands. Although several of these SLC–GPCR 'pairs' have been identified over the past century, there have been recent insights into novel regulatory mechanisms of SLCs that modulate the activation of GPCRs. Chapter 2 describes SLCs that mediate the efflux of ligands, as well as SLCs that grant ligand access to intracellular receptors. Identification of such mechanisms could help to understand disease etiology and target selection for drug discovery.

In Chapter 3, the development of the 'transport activity through receptor activation' (TRACT) assay is reported for the human dopamine transporter (DAT, SLC6A3), which is a drug target for treatment of depression and substance abuse disorders. Dopamine, which is the substrate of DAT, is an endogenous agonist for dopamine receptors and adrenergic receptors. Activation of these receptors on live cells leads to changes in cell morphology, which can be measured in real-time as a change in electrical impedance using the label-

free technology xCELLigence. In two human cell lines with heterologous DAT expression, dopamine-induced activation of the dopamine D1 receptor (D1R) or the alpha-2 adrenergic receptor (α2R) was attenuated as a portion of the extracellular dopamine was taken up into the cell via DAT. Pharmacological inhibition or the absence of DAT restored the apparent potency of dopamine on D1R and α2R. This provided an assay window to measure the inhibitory potency of GBR12909 and cocaine, which are well-known DAT inhibitors. This chapter demonstrates a novel application of a cell-based label-free biosensor, which may be used to identify new DAT inhibitors.

In Chapter 4, the concept of the TRACT assay is extended to the human norepinephrine transporter (NET, SLC6A2), which is an established drug target for a range of psychiatric disorders. Here, NET was overexpressed in an inducible human embryonic kidney 293 (HEK293)-JumpIn cell line, which endogenously expresses the α2R. Three endogenous NET substrates – norepinephrine, dopamine and epinephrine – activated α2R, resulting in a concentration-dependent cellular response. This response was attenuated in the presence of NET for all three substrates and could be rescued by the NET inhibitor nisoxetine. Using norepinephrine as a substrate, the inhibitory potencies of several reported NET inhibitors were determined in the TRACT assay, showing a good correlation with a conventional fluorescent substrate uptake assay. Moreover, the assay was validated in a manual high-throughput screening (HTS) set-up, which suggests that the TRACT assay can be used in the future for screening of NET inhibitors.

The TRACT assay that was developed in Chapter 4 was applied in Chapter 5 to screen a set of compounds that were predicted to be NET inhibitors using a computational modeling pipeline. Similarity networks were used to make a selection of the SLC bioactivity data from the ChEMBL database, which was then used to train a proteochemometric (PCM) model. After further optimization, this model was applied to screen the Enamine REAL database that contains over 600 million make-on-demand molecules. From this screen, the molecules with the highest predicted affinity of 46 chemically diverse clusters were identified, of which 32 molecules were purchased for in vitro testing. Using the TRACT assay, five compounds were found to have submicromolar potencies towards NET. This chapter demonstrates that the TRACT assay could be successfully used to identify and characterize unknown NET inhibitors.

Chapter 6 describes the development of an impedance-based phenotypic assay for the Na+-dependent excitatory amino acid transporters (EAATs, SLC1 family), which mediate the uptake of glutamate and aspartate in the central nervous system and peripheral tissues. Initial attempts were made to set up a TRACT assay for EAAT1, by using HEK293-JumpIn cells with inducible overexpression of EAAT1 and transient expression of the metabotropic glutamate receptor 2 (mGluR¬2). The presence of EAAT1 attenuated the glutamate-induced mGluR2 response, although this response was inconsistently restored by two EAAT1 inhibitors. Interestingly, a receptor-independent glutamate response was observed in cells lacking mGluR2, which could be inhibited by EAAT1 inhibitors UCPH-101 and TFB-TBOA in a concentration-dependent manner. Live-cell imaging revealed that the cells started spreading upon treatment with glutamate, which concurs with the observed increase

in impedance. This response was observed with both L- and D-isomers of glutamate and aspartate, suggesting that it is substrate-independent. Moreover, the response was prevented in the presence of the Na+/K+-ATPase inhibitor ouabain, which indicates that it is ion-dependent. Targeted metabolomics showed a decrease of intracellular taurine levels upon glutamate or aspartate treatment of the cells, which indicates an effect on cell volume. Taken together, these data suggest that substrate uptake via EAAT1 induces cell swelling, which triggers changes in cell morphology that are detected in the impedance-based assay. This distinct 'phenotype' was also observed in a breast cancer cell line with endogenous EAAT1 expression, as well as HEK293-JumpIn cells overexpressing EAAT2 or EAAT3, suggesting that the mechanism is shared between cell types and SLCs. Validation of this assay in a manual HTS set-up confirmed that this phenotypic approach may be used for EAAT drug discovery and holds promise for the study of other transport proteins that modulate cell shape.

In Chapter 7, the phenotypic assay from Chapter 6 was applied to assess the function of disease-associated variants of EAAT1. Using in silico methods, several EAAT1 missense mutations were identified in cancer patients listed in the Genomic Data Commons dataset. Eight of these mutations were selected for in vitro testing based on their close proximity to the substrate and inhibitor binding sites. Moreover, two mutations found in patients with episodic ataxia type 6 (EA6) were included for testing. Substrate responses (glutamate and aspartate) and inhibitory potency of orthosteric (TFB-TBOA) and allosteric (UCPH-101) inhibitors were differentially affected by the tested mutants, suggesting either a gain- or loss-of-function. Interestingly, one of the EA6 mutants – M128R – was found to be 'activated' upon treatment with TFB-TBOA and UCPH-101, which was not observed for wild-type EAAT1 or other mutants. These data demonstrate the ability of the impedance-based phenotypic assay to detect altered functionality in transporter variants, which could substantiate mechanistic studies and aid drug discovery efforts.

Chapter 8 provides a general discussion on the various assays that have been presented throughout this thesis. Moreover, a mechanistic substantiation is provided for the TRACT assay, using two models previously described in literature. The main conclusions are presented and the future of cell-based label-free assays in SLC drug discovery is speculated on. Of note, the Appendix provides two insightful tables that summarize SLC–GPCR pairs that share the same substrate, as well as SLCs that are Na+-dependent. Thus, Chapter 8 and the Appendix provide a starting point for the selection of the next SLC that can be assessed using impedance-based assays. Taken together, the findings in this thesis have unveiled cell-based label-free assays as a novel addition to the SLC toolbox, which may prove to be useful in upcoming drug discovery campaigns.