Chronic pain is a disease area with a significant and unmet need for improved treatments. However, due to the complex nature of pain transduction, pain is a notoriously difficult area for drug discovery, where conventional target-based efforts have largely failed to produce new drugs. In this whitepaper, we present a novel phenotypic screening platform based on our proprietary Cellaxess® Elektra technology. This platform provides an exciting opportunity to efficiently identify new classes of compounds that affect neuronal excitability in primary peripheral pain-sensing neurons, while sparing the CNS and cardiovascular system. Combining these three assays means that important decisions about which compounds to advance to further testing and which to eliminate can be made in a seamless operation much earlier along the discovery process, translating to an increase in efficiency, and consequently a decrease in time and costs.

Introduction

Chronic pain is a major public health problem that is set to increase with an aging, and ever-rising world population. A number of analgesic compounds are already available for the treatment of some pain states, although they are ineffective in between one and two thirds of patients suffering from chronic pain conditions1,2. These drugs also suffer from drawbacks in clinical use, with common side effects including neurotoxicity, dependence and gastrointestinal effects such as constipation3. There is therefore a significant and unmet need for improved analgesic compounds, either based on novel or existing mechanisms.

Chronic (or persistent) pain is a condition where the sensation of pain lasts beyond the term of an injury. In the case of acute (or physiological) pain, detection and transmission of intense and/or damaging stimuli is a vital physiological mechanism that provides protection from injury. However, in a patient suffering from chronic pain these protective mechanisms, and therefore pain perception, generally last a long time after the noxious stimulus has disappeared or the injury has healed.

The pathway of pain transduction4 (Figure 1) begins at the injury site with the activation of specific receptors and voltage-gated ion channels in the nerve endings of peripheral neurons. These neurons then convert noxious stimuli into electrical signals (action potentials), which are propagated to the central nociceptor terminal located in the dorsal horn of the spinal cord. From here, transmission of the pain signal occurs, activating second-order neurons that project to the brain.

There are a number of added complexities to this pain transmission process, with rapid functional changes and long-term regulatory changes at both the peripheral and central levels of the nervous system. The perception of pain is also subject to a process called sensitization5 - a key process in chronic pain states6. An increase in the responsiveness of neurons means that neuronal excitation and pain sensation is achieved with stimuli of lower intensity than normal. In addition, neurons may exhibit an enhanced response to noxious stimuli. An important molecular mechanism for peripheral pain sensitization appears to be through altered neurotrophic signaling, whereby an increase in the concentration of agents such as nerve growth factor (NGF) results in a hypersensitivity to heat and mechanical stimuli7. Ion channels also play an important role in the pain pathway, especially with regard to neuronal excitability. Voltage-gated sodium channels are essential to generate and propagate an electrical signal, with the excitability of a peripheral neuron dictated by the type of channels present, as well as their expression levels and plasma membrane distribution8. These sodium channels are produced in the cell body and transported over relatively large distances to their site of action. Mutations that affect this trafficking process, leading to changes in neuronal excitability, have been shown to underlie some major diseases and play a role in chronic pain9. In addition, the expression and function of ion channels are also influenced by the release of inflammatory mediators, such as NGF, which is a key manifestation of chronic pain10.
Prior to the introduction of target-based approaches in the early 1990s, physiological and phenotypic methods were the foundation of pharmaceutical drug discovery. However, with the completion of the Human Genome Project and growing expertise and technological advances in the fields of molecular biology and genomics, it became easier to study the molecular basis for a disease. This led to the widespread adoption of target-based strategies for drug discovery, enabling rational design of medicines and offering the possibility to test their effects on a specific (often isolated) drug target that is directly associated with a particular disease.

While it was originally thought that target-based approaches would revolutionize the drug discovery process and prove to be far more successful in finding new molecules to advance to clinic, the results have not been as expected. In fact, despite dramatic increases in investment for pharmaceutical R&D, a number of studies have noted a decline in drug R&D productivity\textsuperscript{11-13}, an observation partially associated with the advent of target-based drug discovery methods\textsuperscript{13-17}. Interestingly, during a time when target based approaches encompass the vast majority of drug discovery programs, it has been suggested that phenotypic strategies are responsible for more first-in-class small molecule drugs approved by the U.S. Food and Drug Administration (FDA)\textsuperscript{18}. This highlights an opportunity for a broader acceptance of phenotypic screening methods in drug discovery.

The critical difference between target-based and phenotypic methods concerns their physiological relevance, with a phenotypic study generally being more relevant and less artificial. In contrast, target-based experiments often involve isolated targets and so lack the physiological context of phenotypic strategies. In theory, an ideal drug discovery approach incorporates all processes involved, either directly or indirectly, in a compound’s mechanism of action. This is especially true when considering particular disease types that are highly complex in nature and may involve numerous signaling pathways and processes, and affect various proteins. Conditions affecting the nervous system, including chronic pain, are key examples of enormously complex diseases that may therefore benefit from phenotypic drug discovery.
Due to the complexity of pain transduction, a phenotypic discovery strategy may be well suited for identification of novel pain active substances. A platform where compounds are tested using a systems-based approach in a target-agnostic assay, and where phenotypic changes are monitored would therefore be recommended. Supporting this concept, and based on our profound expertise in pain drug discovery, Cellectricon have developed a phenotypic screening platform for the discovery of compounds that alleviate symptoms of chronic pain. This platform is based on our proprietary Cellaxess Elektra technology, on which we have developed a set of primary cell assays that are highly relevant to chronic pain drug discovery.

The primary screening assay enables rapid characterization of medium-sized compound libraries in a chronic pain model based on peripheral, pain-conducting neurons. This fully validated assay was developed in collaboration with Pfizer, and has been shown to identify pain active compounds with great accuracy. In addition, by using a relevant cell or tissue based model from the beginning, compounds without suitable pharmacokinetic properties are removed early in the discovery process.

When looking to affect either a known or an unknown target in the periphery, as determined by the primary DRG assay, it is important to detect any unwanted effects on the heart or central nervous system (CNS). It is quite possible that, if a compound is active against neurons in the periphery, then it will also be active against other neurons in the CNS. In addition, some molecular targets overlap between the peripheral nervous system and the heart. For example, ion channels are potential drug targets that may have toxicity issues, with subtypes expressed in the heart, CNS and pain processing neurons of the periphery. Because ion channels are considered a key drug target in the case of pain drug discovery, it is important that off-target effects are identified and appropriately investigated.

To complement the primary assay, we have therefore developed two highly relevant toxicology models. The purpose of these assays is to assess whether any of the hit compounds from the primary assay also affect critical off-target systems, such as the heart and CNS. Cardiac and CNS toxicities are two of the top causes for the withdrawal of drugs from the market.

Combining these three assays means that important decisions about which compounds to advance to further testing and which to eliminate can be made in a seamless operation. This triaging process can be performed much earlier along the discovery process, translating to an increase in efficiency, and consequently a decrease in time and costs. Furthermore, these assays can be easily complemented with iPSC-derived hepatocyte toxicity testing and additional toxicity assays to further narrow down the number of hit compounds prior to lead optimization or target deconvolution work. An overview of Cellectricon’s phenotypic screening strategy is shown in Figure 2 and demonstrates how an initial primary screening assay for the periphery combines with two safety assays that focus on other important biological systems.

![Cellectricon's screening cascade.](image-url)
Pilot study

Cellectricon’s Cellaxess Elektra Discovery Platform (Figure 3) provides an opportunity to efficiently identify new classes of compounds that affect neuronal excitability in peripheral pain-sensing neurons while sparing the CNS and cardiovascular system early on in the drug development process. We demonstrate the feasibility of this concept with a pilot screen using a limited LOPAC library (Sodium/Potassium Channel Modulators Ligand Set; LO2220; 68 compounds; Sigma).

The Cellaxess Elektra Discovery Platform combines electric field stimulation (EFS) to trigger cellular activity, with plate-based imaging. The platform enables in-situ manipulation and monitoring of neuronal cell cultures directly in high content analysis-compatible 96- and 384-well microplates, with excellent viability and retained cellular morphology. Calcium imaging is employed to monitor the action potential in excitable cells during the electrical field stimulation. Importantly, the platform enables simultaneous monitoring of both neuronal excitability and synaptic transmission (Figure 3). Accordingly, the Cellaxess Elektra Discovery Platform provides a high-throughput methodology to quantify functional responses in native tissues. The typical assay workflow employed is described in Figure 4.

Figure 3  The Cellaxess Elektra® Discovery Platform.

Compounds

The LOPAC library (Sodium/Potassium Channel Modulators Ligand Set; LO2220; 68 compounds; Sigma) includes subtype selective sodium and potassium channel activators and blockers, Na+/H+ antiport blockers, subtype selective ion pump inhibitors and ion transporter inhibitors.

The library was screened in duplicate at a compound concentration of 10 µM in 0.3% DMSO. Tetracline (30 µM) was included in each experiment as a positive control and for QC and normalization purposes for the primary and CNS safety assays. In the cardiac safety assay, forskolin (10 µM) was included as a positive control and for QC and normalization purposes.
Peripheral DRG assay (“primary assay”)

Dissected ganglia from DRGs of adult rats were dissociated using collagenase followed by careful trituration and centrifugation. To model peripheral sensitization in human chronic pain, we exposed the freshly dissociated neurons to 5 ng/mL NGF. After 72 h in culture in 384-well plates, the cells were stained with a FLIPR Calcium-5 Assay Kit (Molecular Probes) and incubated with reference or test compounds. Stimulation parameters were chosen to yield a reproducible, TTX-sensitive response from the DRG neurons as determined by the assay window between control wells and wells with a high concentration of tetracaine (30 μM). We have previously reported that there is an excellent correlation between effects measured by EFS in DRG neurons using this approach and current clamp, including, for example, sodium channel blockade\(^2^3\). The assay was stable with a Z’ above 0.5 with the established EFS protocol (18 V, 150 pulses, 2 ms pulse duration, 10 Hz). Cultures were incubated with substances for 24 h. Active compounds were defined as having an effect larger than 3 × the standard deviation of the untreated control wells at either of the two independent test occasions. The active compounds were tested in concentration response-format (ten concentrations, ranging from 30 µM to 2.6 nM); and the IC\(_{50}\) value determined (if applicable) on the normalized (%-effect) values using a four parameter logistic regression analysis. Compounds with an IC\(_{50}\) of 20 µM or lower in the primary assay, were progressed to the two safety assays.

CNS safety assay

The synaptic function assay was developed to assess synaptic ion channel activity in a more physiologically-relevant context compared to other readily available methods\(^2^4\). The Cellaxess Elektra Discovery Platform was used to simultaneously stimulate a discrete subset of the neurons in each well. Transient, synaptically mediated calcium elevations in neurons distant from the stimulating electric field were recorded. In this way compounds specifically affecting synaptic function can be readily detected and investigated.

Cortical neuronal cultures were created from embryonic day 18 (E18) mouse brains. The tissue was dissociated by mechanical trituration and cells were plated in 96-well plates. Cells were cultured for 12-17 days in NbActiv4 (BrainBits, UK). Cells were stimulated using single pulses of different voltages (9-18 V), as well as with pulse trains. Compounds with an IC\(_{50}\) of 20 µM or lower in the primary assay (above) were assessed for activity in the CNS safety assay in concentration response-format (six concentrations, ranging from 30 to 0.01 µM). Cultures were incubated with substances for 1 h (during the Calcium 5 staining).

Results

From the highly enriched LOPAC library, we identified 18 compounds as active in the primary DRG assay at 10 µM, corresponding to a hit rate of 26%. Importantly, there was an excellent correlation of the compound activity between the two independent test occasions (Figure 5). In the concentration-response follow-up, the activity could be confirmed and the potency determined for 17 of the compounds (Table 1).

![Figure 5](image)

Compound activity was tested in a single concentration screen on two independent test occasions. The correlation between these two tests was excellent.
Interestingly, some compound classes with expected activity in the DRG assay were inactive. For example, a clinically used sodium channel blocker, mexiletine, is inactive. This is not surprising since its potency is rather low and we screened the library at 10 μM, which is around the lowest effective therapeutic plasma concentration for mexiletine. A more general observation is that both openers and blockers of potassium channels, such as acetylprocainamide and glipizide, are inactive in our model system.

The 17 confirmed active compounds were progressed forward to the two safety assays in parallel (Figure 6). Of these compounds, six were inactive in the cardiac assay and seven were inactive in the CNS assay (Table 1). Taking into account activity in both safety assays, this limited pilot screen thus delivered four validated compounds confirmed as inactive in the two safety assays. For example, the compound clotrimazole had a clear safety margin, as seen in Figure 7. This confirms that we were able to efficiently prioritize a “shortlist” of compounds for further testing in the drug discovery process.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>DRG IC_{50} (M)</th>
<th>CNS assay</th>
<th>Cardiac assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Vanillylnonanamide</td>
<td>6.1 x 10^{-7}</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>Paxilline Penicillium paxilli</td>
<td>6.3 x 10^{-4}</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>9.3 x 10^{-4}</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>Benzamil hydrochloride</td>
<td>1.7 x 10^{-1}</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>3’,4’-Dichlorobenzamil hydrochloride</td>
<td>2.5 x 10^{-8}</td>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>5-(N,N-Hexamethylene)amiloride</td>
<td>7.6 x 10^{-4}</td>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>Phenamil methanesulfonate salt</td>
<td>1.0 x 10^{-3}</td>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>Monensin sodium salt</td>
<td>2.1 x 10^{-6}</td>
<td>Active</td>
<td>Inactive</td>
</tr>
<tr>
<td>PNU-37883A</td>
<td>1.0 x 10^{-6}</td>
<td>Active</td>
<td>Inactive</td>
</tr>
<tr>
<td>Propafenone hydrochloride</td>
<td>7.9 x 10^{-7}</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>Salinomycin hydrochloride</td>
<td>1.0 x 10^{-4}</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>1.4 x 10^{-4}</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>Dequalinium chloride</td>
<td>1.8 x 10^{-4}</td>
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<td>Active</td>
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<tr>
<td>Nigercin sodium salt</td>
<td>4.8 x 10^{-6}</td>
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</tr>
<tr>
<td>Aconitine</td>
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<td>Flecainide acetate salt</td>
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<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>Veratridine</td>
<td>1.4 x 10^{-3}</td>
<td>Active</td>
<td>Active</td>
</tr>
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</table>

Table 1 Concentration-response follow up experiments confirmed activity and determined potency for 17 compounds. Of these compounds, seven were inactive in the CNS assay and six were inactive in the cardiac assay.

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**Figure 6** The screening cascade triaged 80 compounds, of which 17 were found to have potency in the primary assay. Safety assays eliminated 13 of these compounds, as they had some activity in either one or both of the cardiac and CNS assays, resulting in 4 validated compounds.
Cellectricon’s assay platform provides an opportunity to efficiently identify new classes of compounds that affect neuronal excitability in peripheral pain-sensing neurons but spare the CNS and cardiovascular system early on in the drug development process. The data presented here demonstrate the ability to perform a small drug screen of pharmacologically relevant compounds with sufficient throughput to support modern drug discovery programs, while maintaining relevance to the intact organism. Further, by incorporating native, cellular selectivity assays in the “on line” workflow, we were able to efficiently prioritize a shortlist of compounds for further testing of in-vivo safety/ADME properties before advancing the compounds to clinic (Figure 7).

We have thus exploited the advantages of using native systems (as opposed to over-expressing systems) both in the primary assay and in the safety assays. These assays take into account changes in neuronal excitability, network function and plasticity as well as the ability to follow pace in the iPSC-derived cardiomyocytes. We believe that when studying complex events, a “functional” screening approach is superior to typically simplified and over-expressing target-based assays. The use of this approach ensures that we can detect broader effects on the heart and CNS cells compared with, for example, hERG and other ion channel screening in over-expressing cell lines.

Conclusions and outlook

An additional important aspect to the Cellectricon platform is the possibility of designing assays with cells of human origin, including iPSCs, for the CNS and pain assays. This ensures that we identify compounds acting at the human counterparts of the targets present in the assays, enabling drug development using patient-specific cultures. Neuronal cell types derived from patient iPSCs make it possible to screen for candidate drugs that will slow down or reverse symptoms of nervous system diseases. Such efforts have previously been made with iPSCs derived from patients with amyotrophic lateral sclerosis (ALS) and Parkinson’s disease. The results of these studies have led to the identification of substances that can reverse ALS symptoms and rescue alpha-Synuclein-induced neuronal toxicity, respectively.

As such, the Cellectricon platform is a valuable tool for functional phenotypic screening campaigns using patient-derived iPSCs and disease-specific neurons derived from these cells. As discussed above, this supports the screening of drugs that can directly alter the function of disease-affected neurons in specific nervous system diseases. This includes assays that monitor neuronal excitability in cell-based models, such as epilepsy, or synaptic transmission in models of Alzheimer’s disease. By offering all assays in the phenotypic screening platform with human origin, from primary assays to safety assays, we increase the predictive power of our native assays even more.

Importantly, the four identified agents all have established connections to pain:

- The antimycotic clotrimazole has been shown to be a ligand at TRP channels, and an unwanted side effect of clotrimazole treatment is burning pain.
- The BK channel blocker, paxilline, has been shown to mimic NGF treatment and sensitize neurons to bradykinin application. Also, paxilline-sensitive currents in cutaneous neurons from inflamed rats deactivate significantly more slowly than those from naïve rats.
- Benzamil has documented antinociceptive effects in several pain models through the inhibition of acid-sensing ion channels and has been put forward as an alternative pharmacological tool in the management of neuropathic pain.
- Vanillylnonanamide is a synthetic capsaicin, which is the prototypical agonist of TRPV1 and the pungent compound in chili peppers.

Figure 7 The compound clotrimazole has a clear safety margin, with activity in the primary assay and no activity in the cardiac and CNS safety assays.
References

3. Honore, P. and Jarvis, MF. Acute and chronic pain, in Comprehensive and Medicinal Chemistry II. Eds. Triggle, DJ and Taylor, JB; Elsevier, Oxford. 327-349