Cell-Based High Throughput Screening Approaches for the Identification of Cytoprotective Compounds

PhD Thesis

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1. INTRODUCTION

Cell-based high-throughput screening (C-HTS) refers to a process in which large numbers of compounds are tested (i.e., screened) in various cell-based models of disease, in order to identify compounds that modulate the process in a desired fashion. For instance, in models of cell death or cell injury, this approach can be applied to identify compounds that exert cytoprotective effects. Using the C-HTS approach, the cell-based assays serve to mimic the complexity of a disease; for instance by simultaneously activating multiple cellular pathways. Drug discovery through C-HTS could significantly decrease the time and cost of new drug development.

The approach of C-HTS has matured over the past two decades into an indispensable part of drug discovery and basic research in the pharmaceutical and biotechnology industries as well as in academic groups. The C-HTS process can utilize different molecular libraries, industrial-scale robots, and sophisticated automation. The goal of C-HTS is to generate cytoprotective chemical structures (leads). After the process of lead optimization and, in some cases, target identification, the most promising candidate can enter the in vivo experiments and further preclinical-clinical studies to become an experimental compound, on its way to potentially become a new therapeutic candidate. The emerging compounds can be also used, as probes to address various biological questions in basic research.

The CellScreen Applied Research Center that belongs to the Semmelweis University, Budapest is one of the academic screening centers in Hungary. Over the last three years, our research group successfully developed three cell-based HTS models: in vitro acute myocardial infarction, in vitro acute tubular necrosis and in vitro insulin resistance models. Furthermore, the development of our in vitro diabetic vascular complication model is ongoing. Approximately 20,000 molecules were screened in each model. The test compounds came from generic libraries and original libraries. These original libraries were created via a combinatorial chemistry approach by AMRI Hungary (formerly ComGenex, a subsidiary of AMRI USA). However, my dissertation focuses only on the generic molecular libraries, especially the LOPAC generic library of 1280 pharmacologically active compounds.
2. AIMS/HYPOTHESIS

The principal aim of the present thesis was to establish the C-HTS methodology in the setting of an academic laboratory in order to screen compound libraries and to identify novel cytoprotective agents and/or to discover new applications of generic compounds, followed by their characterization.

The specific aims were as follows:

- To develop two C-HTS assays: *in vitro* cell-based assays to model myocardial and renal ischemia reperfusion injuries.
- To screen a library of 1280 pharmacologically active compounds (LOPAC) for validating the previously established C-HTS assays and determining compounds with cytoprotective effects.
- To investigate the mode of actions of the cytoprotective molecules emerging from the screens.
3. METHODS

3.1. Cell cultures

H9c2 rat heart myoblast and NRK normal rat kidney epithelial immortalized cell lines were used and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Biochrom) supplemented with 10% fetal bovine serum (Invitrogen), 4 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

In the in vitro oxidant-induced myocyte cell death model H9c2 cells were plated into 96-well tissue culture plates and cultured at 37 °C at 5% CO₂ atmosphere. After a 7-day-long culturing period cardiomyocytes became differentiated providing a well-characterized phenotype. Cardiomyoblasts from passage numbers 40-60 were used for the assays.

In the screening assay of in vitro acute tubular necrosis also NRK cells were plated into 96-well tissue culture plates and cultured for 3 days at 37 °C at 5% CO₂ atmosphere to form a confluent monolayer. NRK cells from passage numbers 10-25 were used for the experiments.

3.2. LOPAC generic library

The Library of 1280 Pharmacologically Active Compounds (LOPAC 1280) includes drug-like molecules in the field of cell signaling and neuroscience (e.g. antibiotics, apoptosis, calcium signaling, gene regulation and expression, ion channels, lipid signaling, multi-drug resistance, neurotransmission, phosphorylation). The compounds were dissolved at 10 mM in dimethyl sulfoxide (DMSO) and dilutions were made either in DMSO or phosphate buffered saline (PBS, pH 7.4) to obtain 0.5% DMSO in the assay volume. The final concentration of the drugs was set as 3-30-50 µM in the screening assays.
3.3. Materials

For the \textit{in vitro} myocardial ischemia-reperfusion screening model, the potent poly-ADP-ribose polymerase (PARP) inhibitor, PJ34 was purchased from Calbiochem. Chloro-APB hydrobromide and bromo-APB hydrobromide were obtained from Sigma-Aldrich. These compounds were dissolved in DMSO and phosphate buffered saline (PBS, pH 7.4) to obtain 0.5\% DMSO in the final assay volume for the experiments.

For the \textit{in vitro} acute tubular necrosis screening assay the adenosine, inosine, the adenosine receptor antagonists (CDPX, CSC, alloxazine and MRS 1523), agonist (IB-MECA), furthermore the adenosine deaminase inhibitor, EHNA were also acquired from Sigma-Aldrich. The adenosine kinase inhibitor, ABT 702 and the PARP enzyme inhibitor, PJ34 were purchased from Calbiochem. The receptor antagonists, as well as the A3-receptor agonist IB-MECA, ABT 702 and PJ34 were dissolved in dimethyl sulfoxide (DMSO) and final dilutions were made in phosphate buffered saline (PBS, pH 7.4) to obtain 0.5\% DMSO in the assay volume. EHNA was dissolved in distilled water. Adenosine and inosine were dissolved in DMEM without glucose.

3.4. Cell viability assays

3.4.1. MTT cell viability assay

To estimate the number of viable cells and viability \% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/ml and cultured at 37 °C at 5\% CO\textsubscript{2} atmosphere for 1 hour in H9c2 and 3 hours in NRK cultures. Cells were washed with PBS and the converted formazan dye was dissolved in isopropanol and measured at 570 nm with background measurement at 690 nm on a Powerwave reader (Biotek, Winooski, VT). A calibration curve was created by measuring the converting capacity of MTT of serial dilutions of both NRK and H9c2 cells. The viable cell count was calculated using Gen5 data reduction software.
3.4.2. Alamar Blue cell viability assay

Alamar Blue viability assay was only used in the NRK cell-based acute tubular necrosis model. Alamar Blue (resazurin, 7-hydroxy-3H-phenoxazin-3-one-10-oxide) was added to the cells at a final concentration of 10 µg/ml and cultured at 37 ºC at 5% CO₂ atmosphere for 3 hours and the fluorescence signal was measured on Synergy2 (Ex/Em: 530/590nm) (Biotek, Winooski, VT). A calibration curve was created using serial dilutions of NRK cells. The viable cell count and viability % were calculated using Gen5 data reduction software.

3.5. LDH cytotoxicity assay

Cell culture supernatant (30 µl) was mixed with 100 µl freshly prepared LDH assay reagent to reach final concentrations of 85 mM lactic acid, 1040 mM nicotinamide adenine dinucleotide (NAD), 224 mM N-methylphenazonium methyl sulfate (PMS), 528 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and 200 mM Tris (pH 8.2).

In the screening assays the changes in absorbance were either read kinetically at 492 nm for 15 min (kinetic LDH assay) or plates were incubated for 15 min and read at 492 nm with background measurement at 690 nm (endpoint assay) on a monochromator based reader (Powerwave reader, Biotek, Winooski, VT). LDH activity was expressed as percent values of \( V_{\text{max}} \) (for kinetic assay in mOD/min) or delta OD (492-690 for endpoint assay) of postive control wells (100%), with negative controls receiving only vehicle as blank (0%).

3.6. Further methods for investigating the cytoprotective effects of ’hit’ molecules

We applied further methods for investigating the mode of actions of the emerging ’hit’ molecules (APB-hydrobromide molecules, adenosine and inosine) from LOPAC generic compounds library screen on our two C-HTS assays (in vitro cell-based assays of myocardial and renal ischemia reperfusion injuries).
The effects of APB-hydrobromides molecules on the activity of poly(ADP-ribose)polymerase enzyme were examined by PAR western blot analysis and cell-free colorimetric PARP activity assay.

In our *in vitro* acute tubular necrosis model the cytoprotective effects of adenosine and inosine were investigated with the administration of adenosine receptor antagonists and agonist. Furthermore, the role of receptor-independent pathways in the mode of actions of adenosine and inosine were also tested with the inhibitors of adenosine deaminase and adenosine kinase enzymes. Finally, the intracellular ATP contents were determined by a commercially available assay.

### 3.7. Statistical analysis

Data are shown as mean ± SEM values. One-way ANOVA was used to detect differences between groups. Post hoc comparisons were made using Tukey's and Dunnett's tests. Data were also analyzed with Student's unpaired *t* test. A value of $p < 0.05$ was considered statistically significant. EC$_{50}$ values were calculated using nonlinear regression. All statistical calculations were performed using Graphpad Prism 5 analysis software.
4. RESULTS

4.1. Establishment of an in vitro C-HTS model of myocardial reperfusion injury

The pathogenesis of reperfusion injury of the heart is complex, but it is frequently modeled in vitro using ischemic and re-oxygenated perfused hearts. Our first aim was to develop a reproducible myocyte cell death model as a C-HTS assay. Subsequently, a library of pharmacologically active compounds (LOPAC) was screened in order to validate the cell system and identify candidates with cardioprotective effects. The H9c2 rat cardiomyoblast cell line was chosen to provide a source of cells with a well-characterized phenotype. Because the reperfusion injury that ensues after ischemia, is, to a significant part, mediated by oxidative stress, the cell injury was elicited by the oxidant hydrogen peroxide (a compound that decomposes in tissues to induce secondary oxidative reactions, including the release of hydroxyl radical). Appropriate concentration of \( \text{H}_2\text{O}_2 \) was added to the cells inducing oxidative stress conditions and the principal readout was determining the cell viability by different methods. Pharmacological control of the assay was the group of cells treated with a relevant poly(ADP-ribose)-polymerase (PARP) enzyme inhibitor, PJ34. This agent was chosen because of the activation of this enzyme has a crucial role in the oxidative stress induced myocardial injury, which can be attenuated by the inhibition of this enzyme. This assay proved to model in vitro some biologically relevant aspects of the reactive oxygen species production during myocardial reperfusion injury.

4.1.1. Identified cytoprotective compounds from the LOPAC generic library

In the \( \text{H}_2\text{O}_2 \)-induced cell death model we used 8 different assay designs to identify compounds with cytoprotective potential: 3 and 24 hour incubation periods were applied and test compounds were added at 3 or 30 µM final concentration either 30 min prior to or 30 min after the hydrogen peroxide challenge and screened in duplicates. This design served to decrease the chance of identifying compounds that
directly interfered with the primary oxidant injury induced by hydrogen peroxide (e.g. by serving as antioxidant agents) as we were primarily interested in compounds that work in a post-treatment regimen, and we expected that these compounds would affect downstream processes of oxidant-mediated cell injury.

Chloro- and bromo-APB hydrobromide molecules, D₁ dopamine receptor agonist, resulted in a markedly improved cell survival values at 3 and 24 hours. As cardioprotective effects of D₁ ligands have not been previously described in the literature, and these compounds emerged from the screen with significant cytoprotections in all of our assay conditions, these agents were selected for further investigations.

4.1.2. APB molecules do not exert their effects via dopamine receptors, but act as indirect inhibitors on cellular PARP activity

The cytoprotection of APB hydrobromide was comparable to the protection afforded by the PARP inhibitor PJ34 in the H₂O₂ induced cell death model and only moderate improvement was detected when APB molecules were combined with PJ34. In addition, the cytoprotective effects of bromo- and chloro-APB hydrobromide against H₂O₂ induced oxidative stress were also preserved even if fluphenazine, a D₁/D₂ dopamine receptor antagonist or chlorpromazine, a non selective dopamine receptor antagonist were administered prior to APB hydrobromide treatment.

To test whether PARP inhibition plays a crucial role in the cytoprotective effect of APB molecules, confluent cultures of H9c2 cardiomyocytes were treated with H₂O₂ to induce PARP activation, and poly(ADP-ribose) (PAR) was detected by Western blotting. H₂O₂ induced protein poly(ADP-ribosylation) could be detected at approximately 120 kDa. Both APB molecules (bromo- and chloro-APB hydrobromide) decreased poly(ADP-ribosylation) at 120 kDa, similar to PJ34, nearly to baseline, at 3-30 μM concentrations (Figure 1A). The D₁/D₂ receptor antagonist fluphenazine had no impact on the PARP inhibitory effect of APB hydrobromide (Figure 1B).

Finally we also examined the PARP inhibitory function of APB hydrobromide in a cell-free system. In a direct PARP assay, the PARP inhibitor 3-aminobenzamide reduced poly-(ADP-ribosylation) to 85% and 4% at 2 and 200 μM respectively, whereas
no significant reduction in PARP activity was detected with either chloro- or bromo-APB hydrobromide (Figure 2).
Figure 1. Cl-APB hydrobromide inhibits cellular PARP activation. H9c2 cardiomyocyte were treated with H$_2$O$_2$ for 60 min and PARP activity was measured by western blotting for PAR. Cells were pretreated with Cl-APB hydrobromide for 30 min before the H$_2$O$_2$ challenge at the indicated concentrations (A) or Cl-APB hydrobromide at 10 µM was applied alone and in combination with receptor antagonist fluphenazine (APB+fluphen.) (B). The H$_2$O$_2$ group received H$_2$O$_2$ treatment only; the PJ34 group received H$_2$O$_2$ and PJ34 at 3 µM, and controls received vehicle only. Representative blots and densitometric analysis results are shown. PAR signals were normalized to tubulin signal and are shown as mean ± SEM. *p<0.05 compared to H$_2$O$_2$ group.

![Figure 1](image1.png)

Figure 2. Direct PARP assay. Purified PARP enzyme was pre-incubated with PARP inhibitor 3-aminobenzamide (3-AB), bromo-APB hydrobromide or chloro-APB hydrobromide at the indicated concentrations, n=6 in each group. PARP was activated with sheared DNA and incubated with labeled (biotinylated) NAD substrate. PARP activity was determined by the measurement of incorporated labeled (biotinylated) ADP-ribose residues and is expressed as percent values of maximum PARP activity (CTL, without PARP inhibitor, n=6). Mean ± SEM are shown. *p<0.05 compared to CTL group.

![Figure 2](image2.png)
4.2. Establishment of an in vitro C-HTS model of acute tubular necrosis

Our research group established an in vitro model of acute tubular necrosis (ATN) in NRK proximal tubular cells and applied a cell-based high-throughput approach to validate the cell system and to screen a standard library of pharmacologically active compounds (LOPAC) in order to identify drugs with cytoprotective properties. As acute tubular necrosis develops on the basis of severe kidney ischemia and hypoxia and primarily damages the kidney epithelial cells, we have set up an assay in cultured kidney epithelial cells in order to mimic these conditions. During the assay, cells were subjected to combined oxygen glucose deprivation (COGD) using hypoxia chambers, followed by re-oxygenation. During the re-oxygenation phase the supplies of oxygen and glucose were restored for the cells. The principal readout was the determination of cell viability by several different methods (similar to the approach employed in our previous oxidant-induced cardiomyocyte-based model). Our group aimed to model the oxygen and glucose deprivation as easily as possible, without any irreversible chemical modification and blockade of mitochondrial function. For this purpose we employed a ‘true’ oxygen and glucose depletion. This served to represent the ischemic period of ATN, when removal of the blood flow blocks the supply of oxygen and nutrients and the elimination of metabolic waste products. The re-oxygenation phase in NRK cultures mimicked the reperfusion part of ATN in which the restoration of blood flow consequently supplies again the oxygen and nutrients to the renal tissue.

4.2.1. Screening results of the LOPAC library in the NRK cell-based ATN model

The LOPAC library was screened in an in vitro model of ATN using a 16-hour-long hypoxia protocol, combined with 4 hours of re-oxygenation. Compounds were found to be ineffective when they were administrated at low concentration (3-30 µM) after the hypoxia insult and prior to the re-oxygenation phase. Therefore, compounds were administered at 50 µM final concentration before hypoxia induction and screened
in duplicates. Test compounds were removed from the screen during the wash-out that preceded the reintroduction of glucose and the re-oxygenation period. Adenosine and inosine were identified as compounds with the most pronounced cytoprotective effects during the screen. Several additional compounds have exerted cytoprotective effects or trends towards cytoprotection in the current assay, but the overall degree of protection was inferior to the effect of adenosine and inosine. Therefore, we have conducted a subsequent integrated pharmacological characterization of these molecules, in order to investigate their cellular mode of action.

4.2.3. Pharmacological characterization of the effects of adenosine and inosine in an in vitro ATN model

We have established a dose-response comparison between the cytoprotective effects of adenosine and inosine, by testing each compound in a concentration range of 1 µM - 1 mM. Adenosine reached the maximum cytoprotective effect at 100 µM, while inosine was found less potent with a highest protection detected from 300 µM and above. Adenosine completely abolished the cellular LDH release at 30 µM, while inosine had similar effects at 100 µM (Figure 3). An EC₅₀ value of 14 µM was calculated for adenosine and 43 µM for inosine based on the viability values obtained by the MTT assay.
Figure 3. Adenosine and inosine exert cytoprotective effects in a dose-dependent manner. Confluent NRK cultures were subjected to combined oxygen-glucose deprivation (COGD, n=96) for 16 hours followed by a 4-hour-long re-oxygenation period. Controls (CTL, n=48) exposed to hypoxia in complete culture medium and showed similar viability and LDH release to cells maintained under normoxic conditions. Viability was determined after 4 hours of recovery by the MTT assay (A, C) and LDH activity was measured in the supernatant (B, D). Adenosine (ADE, n =9) and inosine (INO, n =9) were applied at 1, 3, 10, 30, 100, 300, 1,000 µM. Data are shown as mean ± SEM. #p<0.05 compared to CTL, *p<0.05 compared to COGD.

As both adenosine and inosine have impact on cell surface adenosine receptors (A₁, A₂A, A₂B, A₃) we observed the potential involvement of adenosine receptors in the protective effects of inosine and adenosine. We conducted pretreatments with the adenosine receptor antagonists (aloxazine, CSC, CDPX and MRS 1523) prior to administration of cytoprotective concentrations (30 µM or 300 µM) of adenosine and inosine. CSC and MRS 1523, but not the other antagonists partially inhibited the
protective effects of adenosine and inosine, suggesting the involvement of $A_{2A}$ and $A_3$ adenosine receptors in the cytoprotective action of inosine. The inhibition displayed by the $A_3$ adenosine receptor antagonist (MRS 1523) was more pronounced than the effect of the $A_{2A}$ receptor antagonist (CSC); the latter compound only afforded a slight reversal of the effect of inosine in the MTT assay and did not affect the LDH release.

Treatment of the cells with the $A_3$ receptor agonist IB-MECA exerted a mild protective effect against COGD induced NRK cellular injury, but its effects were significantly less pronounced than that of adenosine and inosine. These findings are consistent with the hypothesis that the cytoprotective effects of adenosine and inosine during COGD are partially, but not fully mediated by $A_3$ adenosine receptor.

Then we focused on receptor-independent pathways especially the pentose phosphate shunt and adenosine kinase mediated routes in the cytoprotective actions of adenosine and inosine. We have measured the effect of adenosine, inosine alone, and in combination with selected pharmacological enzyme inhibitor agents (EHNA, ABT) on cellular viability and ATP levels (Figure 4). Cells pretreated with 300 $\mu$M adenosine, inosine or glucose maintained significantly higher cellular ATP levels than control cells exposed to COGD. Consistent with the viability data (see above), the adenosine kinase inhibitor ABT 702 (30$\mu$M) and the adenosine deaminase inhibitor EHNA (10$\mu$M) reduced the degree of restoration in cellular ATP levels in cells treated with adenosine during COGD. ABT also reduced the restorative effect of inosine and glucose during COGD.
Figure 4. Adenosine and inosine preserve ATP content of NRK cultures during combined oxygen-glucose deprivation. Confluent NRK cultures were subjected to combined oxygen-glucose deprivation (COGD, n=18) for 16 hours. Cells were pretreated with 300 µM adenosine (ADE), inosine (INO) or glucose (GLUC) 10 min prior to hypoxia induction (n=18, each). The adenosine kinase inhibitor ABT 702 (30µM) or the adenosine deaminase inhibitor EHNA (10µM) were applied 30 min prior to adenosine/inosine and glucose addition and were present throughout the COGD period. Values represent ATP content (picomoles) of 100,000 NRK cells. Controls (CTL, n=18) were exposed to hypoxia in complete culture medium and showed similar ATP content to cells maintained under normoxic conditions (not shown). Adenosine or inosine, applied during normoxia, failed to affect cellular ATP levels (not shown). Data are shown as mean ± SEM (n=18). #p<0.05 compared to CTL, *p<0.05 compared to COGD, +p<0.05 compared to ADE, §p<0.05 compared to INO, • p<0.05 and †p<0.05 compared to GLUC.
5. CONCLUSION

The findings demonstrate that cytoprotective agents, including novel indirect inhibitors of cellular PARP activation (chloro-APB and bromo-APB hydrobromide) and energy-preserving molecules (adenosine, inosine) can be identified with the cell-based high throughput screening methodology.

Our laboratory established two cell-based HTS models such as an oxidative stress-induced cardiomyocyte death model and a renal epithelial cell-based assay of acute tubular necrosis. The identification of cytoprotective molecules by these assays provides evidence for the utility of C-HTS method in the academic research setting. The current methods are applicable for cell-based high throughput screening of various additional compound libraries in order to identify bioactive lead molecules and/or to find disease relevant pathways, which influence complex cellular responses.

From the LOPAC generic library screen APB hydrobromide molecules emerged, as prominent indirect PARP inhibitors, which protected cardiomyocytes from oxidative stress injury triggered by H₂O₂. Further investigations are needed to fully elucidate their protective mode of action in animal models of acute myocardial reperfusion injury as well. We demonstrated first the protective role of APB hydrobromide molecules, a D₁ dopamine receptor agonists, in an in vitro model of myocardial reperfusion injury, suggesting that these molecules beside their important neuromodulatory roles in the central nervous system also act as potential cardioprotective agents.

Adenosine and inosine emerged as remarkable cytoprotective agents from the screening assay involving kidney epithelial cells. Subsequent characterization of their mode of action demonstrated that these compounds can preserve energy by interdependent intracellular pathways of pentose phosphate shunt and adenosine kinase mediated route in NRK cultures subjected to hypoxia. Our results indicate that adenosine and inosine have impact on cellular cell surface adenosine receptors and also play a key role in receptor-independent pathways during anaerobic conditions. Future pharmacological therapy of ATN may be based on the pretreatment with compounds that supply energy for the tubular cells.
6. PUBLICATIONS

Publications closely related to the present thesis


Publications not related to the present thesis


Publications in Hungarian:


Módis K, Kiss L, Szabó C. cGMP phosphodiesterase-inhibitors and their clinical applications. Praxis, 2005 April

Kiss L, Módis K, Szabó C. The role of uric acid and xanthine-oxidase inhibitors in cardiovascular diseases. Praxis, 2005 June