Establishment of a HTS amenable platform for the identification of compounds selectively targeting ABCB1 expressing multidrug resistant cancer

Theses of doctoral dissertation

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Introduction

The effectiveness of chemotherapy is hindered by the primary or secondary resistance of cancer. Mechanisms that are capable to provide protection against structurally diverse and functionally distinct drugs are termed multidrug resistance (MDR). One of the most common form of MDR is caused by the overexpression of ABC-transporters in the cellular membrane of cancer cells. Fueled by the energy of ATP, MDR pumps extrude a wide range of drugs from the cells, keeping the intracellular concentrations of xenobiotics below a cell-killing threshold.

Among these ABC-transporters, ABCB1 (P-glycoprotein, P-gp) has earned the most attention, as P-gp is the major factor of the *in vitro* MDR of cell lines, and also because overexpression of this transporter was linked to poor chemotherapy response in several type of malignancies. Unfortunately, several generations of ABCB1 inhibitors have failed to restore the efficacy of P-gp substrate chemotherapeutics in clinical trials. Therefore, novel ways to treat MDR tumors or to prevent the occurrence of MDR are needed.

One of the novel approaches considers MDR as a targetable trait. This idea dates back to the 1950s, when the phenomenon called collateral sensitivity (CS) was coined, following the discovery of drugs targeting otherwise resistant bacteria or cancer cells.

CS is the consequence of the alterations that are acquired as tumors cells adapt to cytotoxic/cytostatic drugs, and which are associated with potential vulnerabilities. Earlier work has identified compounds that specifically target the CS of MDR cells overexpressing P-gp. Such "MDR-selective" compounds have the potential to eliminate MDR clones from a heterogeneous tumor population. Alternatively, these agents could simply prevent the emergence of MDR conferred by P-gp if administered simultaneously, consecutively or prior to chemotherapeutical treatment.

To date, several substances were shown to preferentially kill MDR tumor cells. However, most of these compounds were identified accidentally, as 'by-products' of phenotypic screens, where the specific contribution of P-gp was not assessed. Moreover, the known entities possess moderate *in vitro* potency (cytotoxicity, MDR-selectivity), and there is no working *in vivo* proof of concept study. To identify further, potent MDR-selective agents, well-designed high-throughput screening of compound libraries against parental and MDR cell line pairs would be highly beneficial.

Objectives

Our overall aim was to establish a screening platform for the robust and high throughput identification of MDR-selective compounds, to find drug-like candidates amenable to lead optimization. We wanted to broaden our understanding on the structure-activity relationships (SAR) of MDR-selective compounds, to understand their mechanism of action, in order to design more potent, pharmacologically active candidates.

Objective #1. Establishment of a standardized cytotoxicity testing system amenable for high throughput screening of compound libraries

#1A. Design of a 3-step compound screening system including primary, confirmatory and secondary screening.

#1B. Introduction of a reagent-free, fluorescent protein based cytotoxicity assay measuring growth inhibition.

#1C. Robotization of the screening procedure; automated data evaluation for both 96 and 384 well plate experiments to increase compound throughput.

Objective #2. Identification and validation of MDR-selective compounds guided by literature data

#2A. Verifying the putative MDR-selective toxicity of compounds reported to preferentially kill P-gp overexpressing MDR cell lines.

#2B. Systematic identification of novel MDR-selective compounds from the NCI DTP drug repository database.

#2C. Defining chemotypes linked to MDR-selective cytotoxicity, based on #2A and #2B.

Objective #3. Screening of focused libraries designed to investigate the chemical space around MDR-selective chemotypes

Materials and methods

Compounds that were reported earlier in the literature to exploit the collateral sensitivity of MDR cells were purchased either from vendors or were gifts from other research groups. Compounds, which were acquired from the DTP's screening library were identified by *in silico* data mining, using the publicly available NCI-60 Cell Line Screen database released in December, 2010. To find putative MDR-selective compounds, we determined the Pearson's coefficient between the pGI_{50} values of each DTP chemical entity measured against the NCI-60 cell line panel and the mRNA expression of ABCB1 of the NCI-60 cell lines. The focused library consisting thiosemicarbazones and their analogues was designed and partly synthesized by Veronika F.S. Pape. Compounds of the library containing flavonoids and thiosemicarbazones were synthesized and provided by Ahcène Boumendjel (Univ. Grenoble Alpes, Département de Pharmacochimie Moléculaire, France). Compounds of the protoflavone library were synthesized and provided by the group of Dr. Attila Hunyadi (SZTE, GYTK, Szeged). Most 8-hydroxy-quinolines were synthesized by the groups of Dr. Tibor Soós (MTA, TTK, Budapest) and Dr. Ferenc Fülöp (SZTE, Szeged), while other analogues were obtained from vendors or acquired from the DTP.

Determination of chemotypes associated with MDR-selective cytotoxicity was based on PubChem's 2D Chemical Structure Clustering Tool, and distances were visualized based on the Tanimoto similarity index. Structures of compounds presented in the thesis were sketched by Marvin (ChemAxon Ltd.).

Cell lines and culture conditions

To assess cytotoxicity, we used a dedicated panel of cancer and immortalized cell lines consisting parental and MDR pairs. Cell lines were maintained either in DMEM (Mes-Sa, KB-3-1, A431, MDCK II and DMS 114, and their derivative cell lines) or in RPMI (OVC-8 DsRed2, NCI-ADR/RES eGFP, HCT-15, KB and KB-VIN). Culture medium was completed with 10% FBS, 5 mM glutamine and 50 units/ml penicillin and streptomycin, except for KB and KB-VIN cell lines, where we added 10% FBS, 25mM HEPES and 100 μg/ml kanamycin to the medium. Cells were periodically tested and resulted negative for mycoplasma contamination.

The fluorescent protein expressing cell lines OVC-8 DsRed2 and NCI-ADR/RES eGFP were created by transfection of the respective expression vectors encoding the fluorescent proteins

DsRed2 and eGFP by Lipofectamin2000 reagent, and were kind gifts from Dr. Michael M. Gottesman. Fluorescent variants of Mes-Sa and Dx5 cells were created using lentiviral transduction of the expression vectors by Dr. Áron Szepesi, Dr. Katalin Német and Nóra Kucsma.

Reagent based and fluorescent protein based cytotoxicity assays

Cytotoxicity assessment was performed on both 96 well and 384 well plates. We used MTT (0.5 mg/ml in PBS), PrestoBlue (resazurin based; diluted to 5-10 % in PBS) and Sulforhodamine B (SRB; 0.4 w/v %) reagents to measure cytotoxicity by following the manufacturer's instructions, with slight modifications. MTT conversion and SRB staining were detected by measuring absorbance on the respective wavelengths, while PrestoBlue conversion was measured by fluorescence detection on the given excitation/emission wavelengths. Reagent-based assays were read by a Perkin Elmer EnSpire multimode plate reader at a single point in a well, as solutions were homogenous. The obtained values from top reading mode were normalized to the untreated (negative) control and to a cell-free (positive) control in the case of MTT and PrestoBlue viability reagents, while in the case of SRB, the positive control value was calculated from wells containing cells treated with cell killing concentrations of drugs, to exclude the error that would be derived from not staining the cell debris. Reagent based (end point) assays were typically applied after 72 h incubation of drugs on cells.

Fluorescent protein based cytotoxicity assays and assay development were performed by using various fluorescent proteins (DsRed2, eGFP, mCherry, mOrange). Due to the uneven 2D distribution of the cells on the bottom of the wells, we applied well area scan mode instead of single point measurement. Values obtained from bottom reading mode were normalized to the untreated (negative) control and to wells, where all cells were killed (similarly to SRB assay), as the relative fluorescent value of this positive control was different from values of cell-free wells containing only medium. As the fluorescent protein based detection was not harmful to the cells, we measured the same plates several times, mostly at 72 h, 96 h and at 144 h.

Cytotoxicity (IC_{50} or GI_{50} values) were obtained by sigmoidal curve fitting by the GraphPad Prism software using the four parameter logistic equation with automatic top and bottom plateau determination. Alternatively, automated data evaluation was performed by our custom program, which was written by Judit Sessler in C#, and half-maximal growth inhibition was calculated based on nonlinear least square regression (nls) of the logistic function.

Monitoring assay performance

Robustness of the fluorescent protein based assay was monitored via the Z'-factor, which is the proportion of the assay dynamic range that is not overlapping with either the positive or negative controls' data variability band (covering 99.7 % of all the possible positive and negative control signals if normal distribution is assumed). Z'-factor was calculated as follows:
$Z'\text{-factor} = [|\mu_{neg} - \mu_{pos}| - 3(\sigma_{neg} + \sigma_{pos})]/|\mu_{neg} - \mu_{pos}| = 1 - [3(\sigma_{neg} + \sigma_{pos})/|\mu_{neg} - \mu_{pos}|]$,
where μ_{pos}/μ_{neg} are the mean of positive/negative controls and $\sigma_{pos}/\sigma_{neg}$ are the standard deviation of positive/negative controls.

Items of the automated screening platform

We installed a screening platform to increase the throughput of compound testing, which consists a Hamilton StarLet automated liquid handling machine and a Perkin Elmer EnSpire multimode plate reader. Methods (programs) for liquid handling automation by the robot were written in the Venus2 software.

Incoming compounds were registered and stored in a systematic way. We created a database, where the amount of compounds, disposition of powders/solutions, etc. were stored, to make them easily accessible for testing. Structural information was also entered, which was connected to the Instant JChem software (ChemAxon Ltd.). Dissolved compounds of the liquid library were stored in 1.1 ml tubes in 96 position racks, where tubes could be collected for the experiments individually, while mother plates (96 well polypropylene trays) had fixed layouts with 36 compounds in 2 concentrations per plate in 100 µl.

Results

1. Establishment of a standardized cytotoxicity testing system amenable for the high throughput identification of MDR-selective compounds

We designed a small molecule screening system that is suitable for HTS purposes to identify potent MDR-selective compounds. Cytotoxicity testing included 3 consecutive steps to screen for overall cytotoxicity (I, primary), dose-dependent cytotoxicity in parental and MDR cells (II, confirmatory), and the robustness of activity across different parental & MDR models (III, secondary). As secondary tests, we used counter assays to exclude assay specific false hits.

An additional aim was to minimize liquid handling steps and to reduce costs. Therefore, I developed a reagent-free, fluorescent protein based cytotoxicity assay. Initially, I determined the linear correlation between cell number and the intensity of the expressed fluorescent proteins detected by the plate reader. In the next step, I successfully followed cell growth, and demonstrated the reliable measurement of growth inhibitory effect of cytotoxic compounds, expressed as GI_{50} values. Optimization of the fluorescent protein based cytotoxicity assay was monitored by the robustness calculated as Z'-factor values. I reached higher Z'-factors (thus more robust assay) when the 2D spatial distribution characteristics of the cells were considered, and instead of a single point measurement, I used well area scan mode available in the EnSpire plate reader's software. Z'-factors were increased also by longer incubation time and higher flash numbers (duration of fluorescent signal detection). I found that evaporation from the outer wells affects cell growth, thus those wells were excluded from the cytotoxicity experiments.

In order to increase the throughput and to test larger compound libraries in search for potent MDR-selective agents, automation of liquid handling was indispensable. Therefore we installed a Hamilton StarLet automated liquid handling machine, which was responsible for the seeding of cells in culture plates, serial dilutions and the transfer of dissolved drugs for primary, confirmatory and secondary screening assays. As the methods operate with fix deck layouts, all plate maps had to be standardized and pre-defined by considering also the possible robot specific pipetting channel movements.

2. Identification and validation of MDR-selective compounds guided by literature data

Several compounds were reported to preferentially kill P-gp overexpressing cancer cells *in vitro*. However, in many cases, the causal link between P-gp and hypersensitivity of MDR cells to CS agents were not examined thoroughly. Therefore we collected several substances to probe

their robustness in killing MDR cells. We tested verapamil, reversin121, TritonX-100, desmosdumotin B flavonoids, rotenone, KP772, Dp44mT and the Pluronic block copolymer P85. We found that except for KP772, the reported compounds did not possess robust MDR-selective activity, and the provoked collateral sensitivity was linked to other factors than functional P-gp.

In contrast, when we extended the systematic datamining of the NCI DTP drug repository, we identified robust, P-gp mediated MDR-selective compounds. Regarding to cytotoxicity and MDR-selectivity, the best performing hit was NSC297366, which was tested against additional cell line pairs, where we further demonstrated its robust selective effect that was independent also of the cytotoxicity assay type we used.

In order to associate chemotypes to the observed MDR-selective effect, we clustered the compounds identified in the DTP database based on their 2D structures. A prominent class contained 8-hydroxy-quinolines (8-OH-Qs), including NSC297366 and 5 closely related analogs. Other structural congeners that were associated with MDR-selectivity were the 1,10-phenanthroline complexes, β-diketones and thiosemicarbazones.

3. Screening of focused libraries designed to investigate the chemical space around MDR-selective chemotypes

We compiled a set of compounds representing the chemotypes that were associated with MDR-selective cytotoxicity. The libraries, consisting of 2160 compounds, were subjected to primary cytotoxicity screening to exclude non-toxic and less active analogues. Four focused libraries were further characterized in confirmatory and secondary screens, built around (i) 8-hydroxy-quinolines, (ii) thiosemicarbazones (TSCs), (iii) TSCs and flavonoids including azaaurones and (iv) protoflavones. The 8-hydroxy-quinolines library was further refined in iterative steps, leading to the identification of highly potent analogs with significantly increased cytotoxicity and MDR-selectivity. By testing various R2-, R5- and R7-substituents of the core 8-OH-Q, we contributed to the understanding of essential structure-activity relationship. The best performing entities, which were highly effective against several MDR lines possessed an R7 of methoxy-benzylamines or tetrahydro-isoquinoline derivatives.

By testing thiosemicarbazones, flavonoids and protoflavones in 3 distinct focused libraries, we observed crucial relationships between structure and CS activity. We found that among TSCs, only β-isatin-thiosemicarbazones (such as NSC73306) elicited a mild but significant P-gp mediated MDR-selective cytotoxicity, while other analogues provoked hypertoxicity against resistant cells independently of transporter expression. When we tested a 156 membered library

of flavonoids (3-aryl-2-quinolones, flavones, aurones, azaaurones, chalcones, xanthones and azaflavones), we found that the azaaurones and chalcones were more toxic than the other groups. While chalcones were unfortunately not hypertoxic to the MDR cell line Dx5, most of the azaaurones possessed a selectivity ratio greater than 2, when the IC_{50}s of the parental and MDR cell lines (Mes-Sa and Dx5, respectively) were compared. In follow up experiments, we investigated if the cytotoxicity of azaaurones were potentiated by P-gp. By applying an ABCB1 transfected cell line, we demonstrated a slight (1.7-fold) but significant MDR-selective cytotoxicity of only one analogue, while other azaaurones were not killing the ABCB1 transfected cells preferably. Probing a protoflavone library returned several entities that were selectively toxic against drug-selected MDR cells compared to their parental lines; however ABCB1 transfected lines were not significantly hypersensitive to any of these substances. Nevertheless, protoflavones could evade MDR conferred by P-gp and killed the cells with a relatively low IC_{50} values, thus can be still potentially used as potent anticancer agents.

Conclusions

The overall aim of my doctoral work was to establish a screening platform for the identification of novel MDR-selective analogs possessing increased toxicity and selectivity.

1. I established and characterized a fluorescent protein based cytotoxicity assay, and proved that it is amenable to screen putative MDR-selective substances in a higher throughput.
2. I found that several compounds reported to target MDR cells do not possess robust MDR-selective activity, suggesting that the provoked collateral sensitivity was linked to other factors than functional P-gp.
3. I extended the systematic datamining of the NCI DTP drug repository. This analysis led to the identification of MDR-selective compounds possessing robust activity.
4. I performed structural clustering of the known MDR-selective compounds in order to associate 2D structures (chemotypes) to the observed MDR-selective effect.
5. By screening the cytotoxicity of 2160 compounds, which were purchased based on the chemotypes that were associated with MDR-selective cytotoxicity, I contributed to the generation of lead (or lead like) compounds.
6. I contributed to the understanding of essential structure-activity relationship of 8-hydroxy-quinolines, which led to the development of more, highly active compounds. The most potent, so far unknown analogues were filed in an international patent application "*MDR-reversing 8-hydroxy-quinoline derivatives*" (application no.: pct-hu2017-050009).
7. I investigated the MDR-selective cytotoxicity of structural congeners of thiosemicarbazones and flavonoids (particularly protoflavones and azaaurones).

Publications related to the thesis

Füredi A, Tóth S, Szebényi K, Pape VF, Türk D, Kucsma N, Cervenak L, Tóvári J, Szakács G. *Identification and Validation of Compounds Selectively Killing Resistant Cancer: Delineating Cell Line–Specific Effects from P-Glycoprotein–Induced Toxicity*. Mol Cancer Ther; 2017, 16(1):45-56.

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