Background

Although considerable progress has been made in treating cancer over the past decade with a gradual decline in cancer death rates, **there are still over 8 million deaths annually from cancer worldwide**. Cures of solid tumors can often be achieved by locally directed therapies such as surgery or radiotherapy, but eradication of metastatic cancer, where treatment relies principally on chemotherapy, is rarely successful. Despite considerable advances in drug discovery, **resistance to chemotherapy** confounds the effective treatment of patients. Cancer cells can become resistant to a single drug or they may acquire broad cross-resistance to mechanistically and structurally unrelated drugs (multidrug resistance (MDR)). ATP-binding cassette (ABC) proteins, present in most living organisms from prokaryotes to mammals, include the best known mediators of MDR. In particular, MDR pump ABCB1 (MDR1-Pgp) actively extrudes many types of drugs from cancer cells, thereby conferring resistance to those agents [1].

I. Accomplishment of research objectives

The major objective of this particular application was to further characterize compounds that I identified to be candidate ABC transporter "sensitizers" [2]. The basic hypothesis was that the pharmacogenomic approach I implemented can be exploited to discover **"MDR-inverse" compounds that selectively target multidrug resistant cancer cells**. It was additionally hypothesized that bioinformatic and biochemical analyses would help to elucidate the mechanism of action as well as the **structural determinants** of such compounds. The results are presented according to the milestones suggested in the original proposal.

Milestone 1: (1) Compounds of interest are identified in DTP's dataset; 50-100 compounds are tested in cytotoxicity experiments; (2) delineating chemical features are defined.

1. Identification of MDR1-inverse compounds

Analysis of an initial subset of the database restricted to 1,429 compounds demonstrated strong correlation, measured by Pearson's correlation coefficient (PCC), between drug sensitivity and Pgp expression across the NCI60 cell panel. A positively correlated thiosemicarbazone derivative (NSC73306) was identified and confirmed to exhibit increased cytotoxicity in Pgp expressing cells [2]. In an effort to screen for compounds that behaved similarly, the correlation **analysis was extended to include a larger dataset of approximately 42,000 compounds** and a subset containing more reliable correlation data of 17,000 compounds. Twenty two compounds from the 17K dataset were first selected by Pearson's correlation coefficient (PCC>=0.4) and by availability from DTP's repository for in vitro screening. Six compounds were found to preferentially inhibit Pgp overexpressing cells in at least three of the five cell line models. Identification of **structural analogs** among the six compounds prompted the attempt to utilize the 42K correlation dataset by complementing the correlation data of candidate compounds with

their chemical structure data. Calculation of statistical significance of correlation by bootstrapping and evaluation of Tanimoto's structural similarity allowed the inclusion of additional candidate compounds below the 0.4 PCC threshold. **The extended screenings revealed a group of 37 compounds ("Discovery-set") demonstrating various levels of growth inhibition against Pgp expressing cells** (submitted)**.**

2. QSAR

Analysis of the DTP dataset

Chemical clustering of the Discovery-set defined two coherent sets. To identify common features that may be responsible for MDR-inverse activity, we generated **three-dimensional pharmacophore models** for each cluster using the most active compounds as templates. **QSAR** analysis identified descriptors that predict MDR1 inverse activity with very high accuracy (r2=0.92) (submitted).

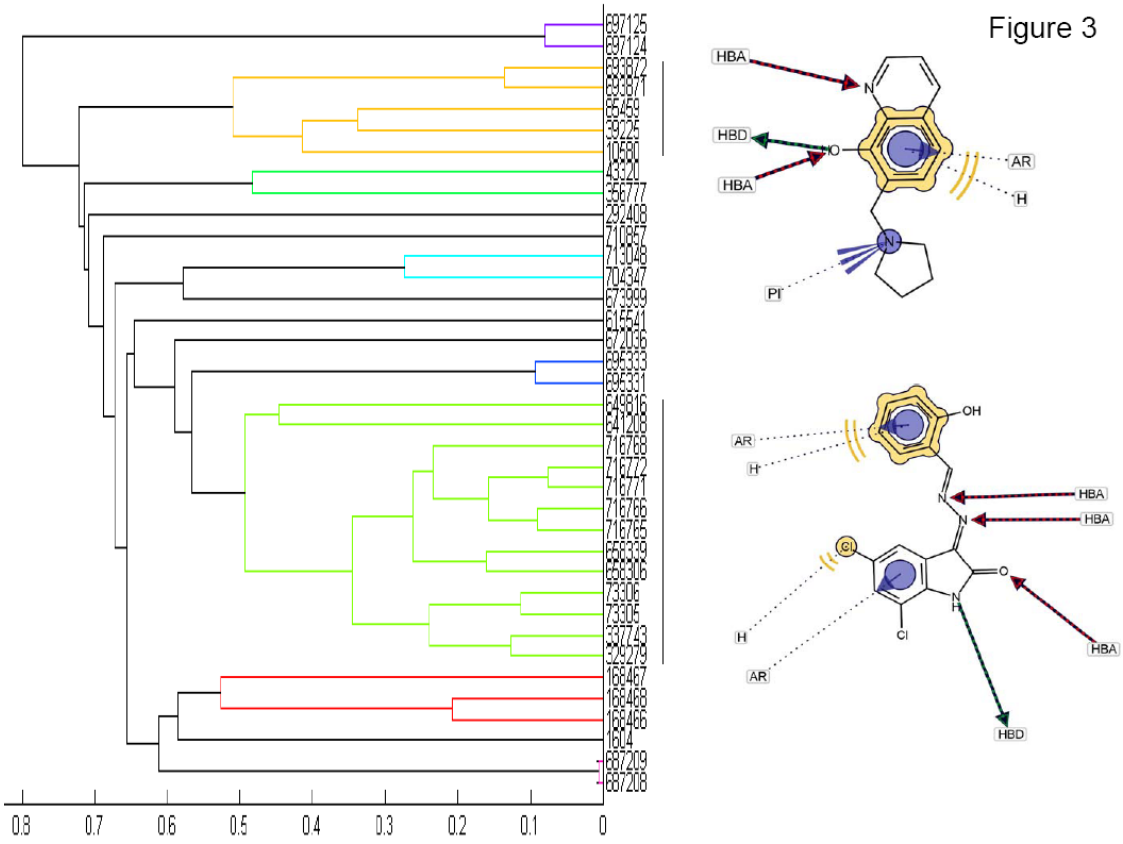

Figure 1. Dendrogram showing the average-linkage hierarchical clustering of the 37 confirmed MDR1-invese compounds. The distance matrix is derived from Tanimoto similarity indices. Numbers represent NSC codes. The two major clusters are colored green ("TSC-cluster") and yellow ("10580-cluster"). Pharmacophore models derived from the two sets are shown. (Taken from the submitted manuscript)

Analysis of focused libraries
Additional work aimed at the delineation of structural features responsible for the MDR1-inverse activity of NSC 73306. A range of **analogs were synthesized** and tested to determine the structural features required for the MDR1-inverse activity of this thiosemicarbazone (ITS). A number of analogs demonstrated improved MDR1-inverse activity over the lead isatin-β-thiosemicarbazone, NSC73306. The MDR1-inverse pharmacophore highlights the importance of aromatic/hydrophobic features at the N4 position of the thiosemicarbazone, and the reliance on the isatin moiety as key bioisosteric contributors for MDR1 selectivity. Additionally, a quantitative structure-activity relationship (QSAR) model that yielded a cross-validated correlation coefficient of 0.85 effectively predicts the cytotoxicty of untested thiosemicarbazones. Together, the pharmacophore and QSAR models serve as effective approaches for predicting structures with MDR1-inverse activity, and aid in directing the search for the mechanism of action of NSC73306 and its congeners [3].

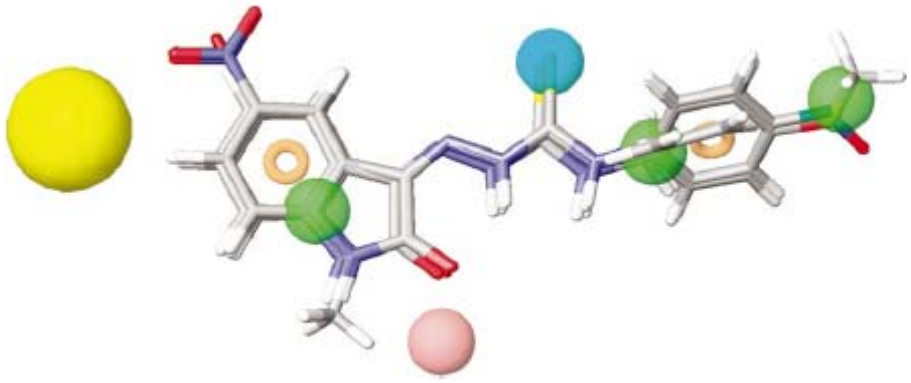

Figure 2. Pharmacophore model derived from the focused ITS library [3].

Milestone 2: Interaction with Pgp

To assess whether the potentiation of cytotoxicity against Pgp-expressing cells is robust, and not cell-line specific, compounds that were available in larger quantities were tested on further multidrug resistant cell lines. Irrespective of the selection drug maintaining Pgp expression, the tissue of origin, and in a genetically engineered (transfected) cell line (NIH 3T3 G185), all compounds showed elevated toxicity in Pgp-expressing cells relative to their parental line, demonstrating that the observed potentiation of cytotoxicity is not restricted to KB-V1 cells and **functional Pgp is the main determinant for selective activity**. In addition, we have found that (i) resistance to NSC 73306 is accompanied by reduced Pgp expression (The analysis of the transcriptional profile associated to resistance against MDR1-inverse compounds is underway); (ii) there is no direct interaction between Pgp and NSC 73306 [4]; (iii) NSC73306 is not more toxic to cells that overexpress other ABC transporters than Pgp (manuscript in preparation). Interestingly, (iv) NSC73306 is a transport substrate for ABCG2 and can effectively

inhibit ABCG2-mediated drug transport and reverse resistance to both mitoxantrone and topotecan in ABCG2-expressing cells (manuscript in preparation).
Overall, NSC73306 seems to be a potent modulator of ABCG2 that does not interact with MRP1, MRP4, or MRP5. Collectively, these data suggest that NSC73306 may be used, due to its dual mode of action, as an effective agent to overcome drug resistance by eliminating P-glycoprotein-overexpressing tumor stem cells [5] and by acting as a potent modulator that resensitizes ABCG2-expressing cancer cells to chemotherapeutics (manuscript in preparation).

Milestone 3: Mechanism of action, preclinical studies

Understanding the mechanism of action behind Pgp-potentiated toxicity would be a major breakthrough and would establish the conditions for a mechanism-based drug discovery research and the development of a molecular target-based research strategy. Although we have gained a deeper insight into the plausible mechanism of action of at least a subset of the MDR-inverse compounds, the underlying physiological principles remain obscure.

Self-organizing maps (SOM) provide a visually compelling clustering algorithm to analyze the relation of drug activity patterns to functional categories representing distinct **modes of action**. To find out if the MDR1-inverse compounds are associated with a specific response category, we projected the Discovery-set on the SOM representing cytotoxicity measurements of the DTP tumor cell screen (http://spheroid.ncifcrf.gov). Strikingly, most of the active MDR1-inverse compounds project to the S-region (linked to nucleic acid metabolism), together with metal chelation complexes and chelators. Typically, metal complexes projecting to the S region show some degree of similarities in both structure and cytotoxic response profiles. To explore the **role of chelation** and metal ions in the activity of MDR1-inverse compounds, experiments were performed with free ligands and chelated complexes. We found that the MDR1-inverse agents possess cytotoxic response profiles similar to their metal chelates, and that the metal type does not appear to significantly affect the activity of these metal chelates, indicating that either they are prodrugs and the active species are their metal chelates formed in situ, or the metal chelates serve as carriers for chelators which themselves are the active drug molecules (submitted).

Drug Development: preclinical studies

In the framework of DTP's Drug Development Program (National Cancer Institute, NIH), a study is underway to evaluate the in vivo anticancer effects of NSC73306. The overall aim of this collaboration is to facilitate preclinical development of NSC 73306. The first task was to develop and validate a sensitive analytical method to quantify the presence of NSC73306 in biological fluids (initially for mouse and human plasma). We characterized the in vitro stability of NSC 73306 in aqueous buffers, plasma and appropriate sample handling and storage procedures. We determined the extent of protein binding, optimized solubility, drug formulation, dosing schedules, and pharmacokinetics.

In all, we have identified a bioavailable formulation that shows antitumor potential in hollow-fiber models [6]. Preliminary lethality/toxicity studies and evaluation of drug efficacy in mice and dogs are underway.

In summary, the goals of the proposal were met: (i) The chemogenomic approach was successfully employed to identify several anticancer drug candidates with enhanced toxicity in otherwise multidrug resistant (MDR) cells (Discovery-set). (ii) Analysis of the Discovery-set in relation to the DTP drug database led to the formulation of initial QSAR and pharmacophore analyses. (iii) Biochemical data coupled with the unsupervised self-organizing map-based clustering of the MDR1-inverse compounds suggested a mechanism of action linked to metal chelation; (iv) preclinical studies evaluating the in vivo effect of NSC 73306 have been initiated.

During the granting period, several papers directly relating to the proposed aims have been published [3-5].

The manuscript summarizing our results obtained with the Discovery-set is currently under review (*Dora Turk, Matthew D. Hall, Benjamin F. Chu, Joseph A. Ludwig, Henry M. Fales, Michael M. Gottesman, Gergely Szakács: Identification of compounds selectively killing multidrug resistant cancer cell*). The results have been presented at a recent national meeting (*Türk Dóra, Matthew D. Hall, Michael M. Gottesman, Szakács Gergely: MDR1-INVERZ CITOTOXIKUS HATÁST MUTATÓ VEGYÜLETEK VIZSGÁLATA, poszter, 39. Membrán-transzport konferencia, 2009).*

In addition, I have coauthored several original papers [7, 8] and review articles relating to the problem of ABC transporters and multidrug resistance [9-12].

II. New objectives established during the course of work and new lines of research.

During the course of the three years of this project, we followed the initial research strategy. The new objectives logically follow the line of drug development. Since we now understand that the initial discovery of a single MDR-inverse agent was only the "tip of the iceberg" [2], our next aim is to aid the preclinical development of the best drug candidates. The relevance of this project to applied research is evident, given the unmet need of conquering multidrug resistant cancer. Based on the promising results obtained in this project, a consortium was formed by academic researchers, renowned experts of drug discovery research and biotech companies working in the field of transporter research. The aim is to target industrial research funds in order to establish the framework for the preclinical development of the most promising MDR-inverse molecules, setting the stage for a fresh therapeutic approach that may eventually translate into improved patient care.

III. Training

The training objective of the OTKA Postdoctoral Fellowship was to ease my **reintegration** to Hungary. While the difficulties I met were unexpected and sometimes

insurmountable, I wouldn't have been able to get established without the valuable contribution of this grant mechanism. During these three years, I published 12 papers with a cumulative impact factor of 85, and I transitioned from a postdoctoral fellow to a group leader in the Institute of Enzymology. Based on the results presented in this report, I was able to attract further national and international funding, as well as talented students and postdoctoral fellows.

III. Relevant publications

1. Szakacs, G., et al., *Targeting multidrug resistance in cancer.* Nat Rev Drug Discov, 2006. **5**(3): p. 219-34.
2. Szakacs, G., et al., *Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells.* Cancer Cell, 2004. **6**(2): p. 129-37.
3. Hall, M.D., et al., *Synthesis, Activity, and Pharmacophore Development for Isatin-beta-thiosemicarbazones with Selective Activity toward Multidrug-Resistant Cells.* J Med Chem, 2009.
4. Ludwig, J.A., et al., *Selective toxicity of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer.* Cancer Res, 2006. **66**(9): p. 4808-15.
5. Turk, D. and G. Szakacs, *Relevance of multidrug resistance in the age of targeted therapy.* Curr Opin Drug Discov Devel, 2009. **12**(2): p. 246-52.
6. Chan KK, J.W., Xie Z, Cheng H, Liu Z, Covey JM, Ludwig J, Szakacs G, Gottesman MM, *Oral bioavailability and pharmacokinetics in CD2f1 mice of NSC73306, an antitumor agent that selectively kills multidrug-resistant cancer cells.* European Journal of Cancer Supplements, 2006. **4-12, 60**.
7. Okabe, M., et al., *Profiling SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify drug uptake transporters.* Mol Cancer Ther, 2008. **7**(9): p. 3081-91.
8. Paterson, J.K., et al., *Human ABCB6 localizes to both the outer mitochondrial membrane and the plasma membrane.* Biochemistry, 2007. **46**(33): p. 9443-52.
9. Hegedus, C., et al., *Ins and outs of the ABCG2 multidrug transporter: an update on in vitro functional assays.* Adv Drug Deliv Rev, 2009. **61**(1): p. 47-56.
10. Szakacs, G., et al., *The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox).* Drug Discov Today, 2008. **13**(9-10): p. 379-93.
11. Sarkadi, B., et al., *Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system.* Physiol Rev, 2006. **86**(4): p. 1179-236.
12. Gottesman, M.M., et al., *Defeating drug resistance in cancer.* Discov Med, 2006. **6**(31): p. 18-23.