Summary of the Ph.D. dissertation

Examination of the ABCB1 transporters in multidrug resistant rat hepatoma cell lines

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INTRODUCTION

The continuous increase of cancer incidences is one of the most important issue of mankind. A key factor in the successful chemotherapy in cancer treatment is the effectiveness of the drug used either alone or in combination with radiotherapy or surgery. Often one of the main reasons of failure during chemotherapeutic treatment of a patients is the so called multidrug resistant (MDR) phenotype of tumors. A number of cellular mechanisms have been described to explain the phenomenon of acquired MDR. The classical multidrug-resistant phenotype of tumor cells is acquired via an increased capability of drug efflux by energy-dependent transmembrane transporters. The transporters involved are members of the ABC (**A**TP-**B**indig **C**asette) protein superfamily. The major physiological function of ABC transporters is to extrude xenobiotics from the cytoplasm, thereby helping cell survival. Grotesquely, cancer cells take advantage of this natural protection mechanism in order to ensure their own survival. For this phenomenon, the increased activity level of the ABC family member ABCB1/MDR1/P-gp protein is responsible. A number of different mechanisms have been described which might result in an increased ABCB1 activity level. In drug resistant cell lines, gene amplification, chromosomal translocation and mRNA-stabilization can contribute to elevated ABCB1 protein levels. On the other hand, changes in the transcription activation of the ABCB1 gene can also play an

important role in this process since several transcription factors, tumor suppressors, oncogenes and epigenetic factors and modifications (histone acetylation, DNA methylation) influence the transcription of the gene.

I used multidrug resistant rat hepatoma cell lines to explore the molecular mechanisms underlying MDR, in particular, I was interested to determine the role of histone acetylation in the developement of multidrug resistant phenotype. In my experiments I used multidrug resistant cell lines which showed medium (col500) and high (col1000) level resistancy. These cell lines were selected from a dexamethasone-resistant hepatoma clone 2 (D12) by increasing the concentrations of colchicine. Previous studies have demonstrated that the drug-selected cells overexpress *Abcb1* mRNAs and display resistancy to structurally unrelated drugs.

AIMS

In order to study the role of different mechanisms, especially histone acetylation on the elevated ABCB1 expression in multidrug resistant rat hepatoma cell lines, I aimed to answer the following questions:

1. Does the elevation of ABCB1a and/or ABCB1b transporter-activity responsible for the development of MDR phenotype? Does the elevated activity of ABCC1 play a role in MDR?

2. What is responsible for the elevated ABCB1 activity
   - the amplification of *Abcb1a* and *Abcb1b* genes
   - the stabilization of *Abcb1a*- and *Abcb1b*-related mRNAs
   - transcription activation of *Abcb1a* and *Abcb1b* genes?

3. What is the relationship between alterations in the transcription of *Abcb1* genes and alterations in the chromatin structure caused by changes in histone modifications?

4. Can *Abcb1* expression be altered by modification of histone acetylation pattern using histone deacetylase inhibitor?

MATERIALS AND METHODS

- Determination of the activity of transporter-proteins by calcein-assay method

- Examination of gene expression by quantitative real-time PCR (QPCR)

- Determination of ABCB1 protein-levels by Western blot analysis

- Comparsion of RNA stability

- Examination of the *Abcb1* regulatory regions by sequencing

- Transfection of reporter plasmids and luciferase activity measurement

- Determination of H3K9 acetylation by chromatin immunoprecipitation

RESULTS

1. Transporter proteins responsible for MDR phenotype

At first, I characterized the transporter activities of the studied cell lines. I found that the activity of the ABCB1 transporter is significantly higher in the drug resistant cells as compared to the sensitive D12 parental cell line. This result is in accord with the observations of those who established the cell lines. Surprisingly, drug selection did not increase, rather decreased the activity of the ABCC1 transporter. Iin drug sensitive D12 cells a higher activity of this transporter was detectable as in the resistant cell lines, in which this transporter was nearly inactivated. In rodent genomes, there are two genes encoding homologues of the major human drug transporter ABCB1. The contributions to drug resistency of the two transporters encoded by these genes were not distinguished previously in the cell lines I used. Therefore I found it important to compare their activities. I found that the activity of ABCB1a was decreased in the drug resistant col500 and col1000 cells as compared to the drug sensitive D12 cell line. In contrast, the activity of ABCB1b was increased in both drug-resistant cell lines as compared to the sensitive cell line. In summary, based on these data I concluded that the ABCB1b transporter has the major contribution to the MDR phenotype of the studied drug-resistant rat hepatoma cells.

2. Several mechanisms contribute to incresed ABCB1 activity

Since high drug efflux activity may result from several mechanisms affecting *Abcb1* gene expression and/or ABCB1 activity, next I compared the mRNA levels of *Abcb1a* and *Abcb1b* by quantitative RT-PCR. I found that *Abcb1a* and *Abcb1b* mRNA levels differed significantly in the parental D12 cell line, being the level of *Abcb1a*-specific mRNA nearly thirty-fold higher than that of *Abcb1b*. In the drug-resistant col500 and col1000 cell lines, increased mRNA levels suggested that both genes were upregulated. Curiously, while the mRNA level of *Abcb1a* was higher than that of *Abcb1b* both in the parental and the resistant cell lines, the increase of *Abcb1b* expression in drug-resistant versus parental cells was considerably higher than that of *Abcb1a.* Determination of ABCB1 transporter protein levels by Western blot confirmed that the resistant cell lines had slightly higher ABCB1b amount compared to the sensitive D12 cells, while the expression level of ABCB1a differed only very modestly between sensitive and resistant cells.

Many factors may contribute to the elevated *Abcb1* mRNA levels in drug resistant cell lines. Among these, one possible reason for *Abcb1* overexpression can be gene amplification. However, I observed only small fluctuations in the quantity of the *Abcb1* genomic regions (this did not reach a two-fold difference) between D12 and the resistant cell lines. Therefore, I concluded that gene amplification alone is an unlikely cause of *Abcb1* overexpression in the studied rat hepatoma cell lines, as the differences in mRNA

levels were much higher than one would expect based on the observed small differences in copy numbers. Another possibility of increased *Abcb1* mRNA levels could be stabilization of *Abcb1* mRNAs in the drug-resistant cell lines. This, however also not a reason for the drug resistancy since I detected only very modest differences in the decay of *Abcb1a* mRNA between drug-sensitive and resistant cell lines. On the other hand, *Abcb1b* transcript levels in actinomycin D-treated col500 and col1000 cells were higher than in the parental D12 cell line at almost each time point tested. However, the increases of mRNA half-lifes in the drug-resistant cells cannot serve as an explanation for the 5-20 times higher mRNA levels detected in these cells.

Since data from neither gene amplification nor mRNA-stability experiments could explain the elevated mRNA-levels, next I compared the transcription rate of *Abcb1* genes. For this I compared the levels of unprocessed *Abcb1a* and *Abcb1b* pre-mRNAs. I detected elevated pre-*Abcb1a* and pre-*Abcb1b* levels in col500 and col1000 cell lines compared to the parental D12 cells. These data therefore suggest that *Abcb1* genes are transcriptionally up-regulated in drug-resistant cells. This could serve as the main contribution to the elevated mRNA-levels in these cells.

3. Histone modifications which are influence the expression of *Abcb1* genes

For transcription factors regulating *Abcb1* expression it is critical to gain access to their binding sites, which is highly affected by the chromatin structure. In recent years, the roles of DNA methylation and histone acetylation in the regulation of *Abcb1* gene expression have been intensively studied. Using drug-sensitive and drug-resistant human breast carcinoma cells, it has been shown that the level of acetylated H3K9 was increased in the promoter region of the intensively transcribed *MDR1* gene in drug resistant cells compare to sensitive ones. Despite the fact that *MDR1* gene expression was considerably higher in resistant cells, surprisingly I found that the acetylated H3K9 levels in the initiator regions of both *Abcb1* genes were similar in the sensitive and resistant cell lines. This suggests that an increased level of acetylated H3K9 at the promoter is not associated with *Abcb1* up-regulation in the col500 and col1000 cells. The level of acetylated H3K9 was higher in the *Abcb1a* initiator region than in the initiator region of *Abcb1b.* This observation correlates well with the amount of the mRNAs transcribed from the genes. These results might suggest that H3K9 acetylation is important for the up-regulation of *Abcb1a* relative to *Abcb1b* in all cell lines, but the H3K9ac levels do not correlate with the expression levels of the *Abcb1* genes in the different cell lines.

4. The effect of histone deacetylase inhibitor treatment on the expression of *Abcb1* genes

There is a widely accepted model, which states that there is a hierarchical relationship between DNA methylation and histone acetylation, being methylation is dominant over acetylation. According to this model, the methylation status of the *Abcb1* genes has to be low in the studied three hepatoma cell lines, because all of them express these genes. Consequently, histone deacetylase inhibitor treatments should cause transcription activation of both genes in these cell lines, as methylation will not interfere with the gene activation resulted from elevated histone acetylation upon HDACi treatment. To test this hypothesis, I treated the cells with trichostatin A, a well-known HDACi. In the case of *Abcb1b*, I detected the expected affect of TSA-treatment: the mRNA level was considerably higher in the treated cells compared to the untreated ones in all cell lines. On the contrary, in the case of *Abcb1a* the mRNA level was unexpectedly lower in the treated cells. A comparison of *Abcb1* pre-mRNA levels in TSA-treated versus untreated cells indicated that the histone deacetylase inhibitor affected the transcription of the two *Abcb1* genes differently: it decreased the expression of *Abcb1a* and in parallel it increased the expression of *Abcb1b* in the parental and the drug-resistant cell lines as well.

SUMMARY

In this dissertation several lines of evidence are provided for the complicated and complex regulation of the overexpression of ABCB1 transporters, which are play an important role in the development of MDR phenotype of cancer cells. In the studied rat hepatoma cell lines, *Abcb1a* and *Abcb1b* mRNA levels increased significantly with drug resistancy compared to the parental drug sensitive D12 cell line. I concluded that gene amplification alone is an unlikely cause of *Abcb1* overexpression, however, in the case of *Abcb1b*, an increase of mRNA half-life might contribute to elevated mRNA levels in the drug-resistant cells. *Abcb1* genes are transcriptionally up-regulated in col500 and col1000 cells, which could serve as the main contribution to the elevated mRNA-levels in these cells. H3K9 acetylation has an important role for the up-regulation of *Abcb1a* relative to *Abcb1b* in all cell lines, but the H3K9ac levels do not correlate with the expression levels of the *Abcb1* genes in the different cell lines. The histone deacetylase inhibitor treatment affected the transcription of the two *MDR1* genes differently in the studied cell lines: it decreased the expression of *Abcb1a* and in parallel it increased the expression of *Abcb1b*, however, it did not significantly influence the drug efflux activity of rat hepatoma cells.

LIST OF PUBLICATIONS

Publication related to the thesis:

Ádám Sike[1*], Enikő Nagy[1*], Balázs Vedelek[1], Dávid Pusztai[1], Anikó Venetianer[3] and Imre M. Boros[1, 2, #] **mRNA levels of related *Abcb* genes change opposite to each other upon histone deacetylase inhibition in drug-resistant rat hepatoma cells** PLOS ONE 9:(1) pp. 1-12. (2014) **IF: 3,730**

Other publications:

Ildikó Huliák[1*], **Ádám Sike**[1*], Sevil Zencir[2*] and Imre M. Boros[1, 3, #] **The objectivity of reporters: interference between physically unlinked promoters affects reporter gene expression in transient transfection experiments** DNA and Cell Biology 31:(11) pp. 1580-1584. (2012) **IF: 2,344**

Sevil Zencir[1], **Adam Sike**[†], Melanie Dobson[‡], Ferhan Ayaydin[§], Imre Boros[†, ‖, 1], and Zeki Topcu[¶,1] **Identification of transcriptional and phosphatase regulators as interaction partners of human ADA3, a component of histone acetyltransferase complexes** BJ Gene 450:(2) pp. 311-320. (2013) **IF: 4,654**

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