Anticancer effects of Ribavirin and Inositol hexaphosphate: exploring the cellular mechanisms and the gene expression patterns

PhD thesis

by

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INTRODUCTION

Cancer is a multistep process which can largely be conceived as a consequence of genomic catastrophes resulting in genetic events that change physiologic function of a normal cell. During the progression of the malignancy, cancer cell populations evolve through sequential genetic and epigenetic changes, allowing them to escape the normal homeostatic controls. Malignant phenotype is largely caused by genomic events that activate oncogenes or inactivate tumor suppressor genes. Alterations in expression of protooncogenes and tumor suppressor genes play a key role in oncogenesis. Dysfunction of their protein products leads to abnormal regulation of signaling pathways, which control the cell cycle, apoptosis, genetic stability, cell differentiation, and morphogenetic reactions.

Complexity of malignant phenotype includes enzymic and metabolic imbalance of cancer cells. A ‘‘comprehensive biochemical strategy of cancer cells’’ was revealed by Weber and coworkers (1983), thus introducing a rational approach for the design of enzyme and metabolism pattern-directed anticancer chemotherapy. According to this concept, the increased capacity of the guanylate synthetic pathway provides an advantage for growth and progression of tumors. Inositide-5'-monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme of GTP biosynthesis and a key component in the biochemical program of cancer cells; therefore it was suggested as a sensitive target for cancer chemotherapy. Whereas the biochemical programs disturbed by the clinically used IMPDH inhibitors are well described, the impact of the compounds on molecular level is poorly understood.

Ribavirin (1-ß-D-ribofuranosyl-1,2,4,-triazole-3-carboxamide) was developed as an anti-viral agent against several DNA and RNA viruses. Soon after the discovery of the broad-spectrum antiviral activity of Ribavirin, the drug was proposed as a specific inhibitor of IMPDH blocking the enzyme at the IMP-XMP binding site. Our previous studies demonstrated that its IMPDH inhibitor counterpart, tiazofurin could result in both a differentiation program and apoptosis and reduce the expression of the MYC and RAS genes. Taylor and co-workers provided evidence that treatment with Ribavirin has very little, if any, effect on gene expression on normal blood (PBMC) cells. Kentsis and colleagues showed that Ribavirin treatment can act through the inhibition of translation but has no

effect on either the chromatin structure or the transcription of some oncogenes. Ribavirin binds to eIF4E at the functional site used by 7-methyl guanosine mRNA cap at low micromolar concentrations. With this unforeseeable effect of Ribavirin, it can efficaciously reduce the levels of oncogenes, such as cyclin D1 and suppress eIF4E-mediated oncogenic transformation of murine cells in vitro and of tumor growth of eIF4E-dependent acute myelogenous leukemia cells derived from human patients. The purpose of our investigation was to provide evidence on the anticancer effects of Ribavirin and to gain a deeper insight into the molecular mechanisms of its antiproliferative action in K562 cells.

Inositol hexaphosphate (IP6) is a naturally occurring polyphosphorylated carbohydrate, existing ubiquitously with high concentrations in cereals and legumes.
IP6 has been reported to have significant in vivo and in vitro anti-cancer activity against numerous tumors involving colon, prostate, lung, liver and breast. IP6 exerts its anti-cancer activity by entering into cellular inositol hexaphosphate pool and affecting common cellular signal transduction pathways. However, the molecular mechanism of the anticancer effect of IP6 has not been fully elucidated.

There has been efforts so far to test the bulk changes of gene expression in K562 pathways as a result of 5mM exogenous IP6 treatment. Microarrays, containig 1176 human cDNA fragments revealed significant downregulation of essential protooncogenes as well as upregulation of transcripts involved in apoptosis and differentiation after 24 hours treatment presented by Deliliers and co-workers. With the thorough expression analysis of the selected pathways we wished to decipher the important pathways by which IP6 exerts its antineoplastic effect by using K562 cell line as a model system. In a continuing effort to evaluate safer and more effective forms of therapy, we investigated the effects of IP6 on the gene expression pattern in leukemia cells.

AIMS OF THE STUDY

The molecular mechanism behind the anticancer effect of the Ribavirin and the IP6 have not been fully elucidated. Based on the initial findings, the aims of our study were to explore the cellular mechanisms and the gene expression patterns in leukemia cells following drug treatments.

❶ What is the best method to examine the gene expression signatures in cell cultures after the treatment with Ribavirin and IP6? A standard method had to be optimized and evaluated in order to gain the most accurate and sensitive technology for the further gene expression studies.

❷ The purpose of our investigation was to provide information on the cellular and molecular changes driving the anticancer effects of IP6 and to gain a deeper insight into the gene expression alterations accompanying the antiproliferative action.

❸ The aim of our study was also to explore the cellular and molecular changes leading the anticancer effects of the Ribavirin as a single drug. It was intriguing to gain a deeper insight into the gene expression alterations of its antiproliferative action.

MATERIALS AND METHODS

1. Cell cultures and growth inhibition assay

- MCF-7 breast cancer, HepG2 human hepatocellular carcinoma, OVCAR-5 ovarian cancer cells and the BCR-ABL positive human leukemia cell line K562 were treated with 1, 10, 20, 50, 100 and 150 μM Ribavirin.
- K562 cells were treated with 0, 0.5, 1, 2.5, 5, and 10 mM IP6.
- For measurement of toxicity, cell viability was monitored daily by trypan-blue dye exclusion test at each concentration up to five days.
- The number of surviving cells was used in a regression curve analysis to determine the IC_{50} values at different timepoints.
- One thousand living cells per sample were analyzed using light microscopy for appearance of morphological signs of apoptosis and differentiation in K562 cell cultures.

2. Gene expression studies

- We optimized the parameters of Northern analysis and radioactively-labeled RNA was created by in vitro transcription.
- MYC and GAPDH gene expresseion were measured by Northern analysis and RPA technology using 10 μM Ribavirin treatment on K562. Additionally, gene expression pattern were detected by real-time PCR and Taqman technology (18S rRNA was used as an internal control).
- Whole Human Genome Oligo Microarray studies were done according to the manufacturer's instructions (Agilent; 60mer chip representing 41K human genes) after treatment either with Ribavirin (15μM) or IP6 (750 μM; 5 mM).
- To confirm microarray results, validated TaqMan PCR assays for 9 previously selected genes and the major BCR-ABL transcript were performed on K562 cells treated with 15 μM Ribavirin for 12 hours on an ABI Prism 7900HT Sequence Detection System.
- To understand the characteristic gene expression effects due to the IP6 treatment on K562 cells, Taqman PCR validations were carried out on selected subset of 10 genes for 12 hours.
- Gene expression was measured at three timepoints (3, 12 and 48 h) in both Ribavirin treated (50 μM) and untreated cells. Arrays with four sets of 96 genes were used. Each

reaction was carried out using 0.5–1 ng mRNA as template. Primer/probe sets were chosen from the validated collection of TaqMan Gene Expression Assays. The Q-PCR runs were performed on an Applied Biosystems 7900HT instrument. For data normalization the geometric mean of 2 control genes (RPLP0, β2-microglobulin) was applied. The normalized gene expression was compared to that of untreated cells as calibrators at each time point using the $2^{-\Delta\Delta CT}$ method. All data analysis was performed using the StatMiner software (Integromics).

- Database and Data Analysis: Sequence Detection Software v.2.1; PANTHER v2.5 (Applied Biosystems), ImageJ v1.39 and JcolorGrid v1.57 softwares.

RESULTS

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- We optimized the conditions of the Northern analysis and RPA technology.
- Decreasing MYC expression was detected by Northern analysis, RPA method (GAPDH control) as well as by Taqman Q-PCR (18S rRNA control) after Ribavirin treatment (10 μM).
- Weighing the merits and demerits of the different methods (Northern analysis, RPA technology, microarray, single-gene Q-PCR assays and TLDA) used in our studies, TLDA was proved to best for our purposes on the basis on its specificity, low reagent consumption, standardized and customizable formats. This technology allows multiple targets to be analyzed per sample with very few pipetting steps, streamlining reaction set-up time and eliminating the need for liquid handling robotics.

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- K562 cells were treated with 0, 0.5, 1, 2.5, 5, and 10 mM IP6 respectively and cell survival ratio was monitored daily up to 5 days. Tendency in decreasing of cell survival compared to the non-treated cells was noticed in a time- and dose-dependent manner. We choose IC_{50} dose corresponding to 120 hrs (750 μM), which confers physiological concentration and 24-48 hrs (5 mM), a pharmacological concentration for further treatment analysis. The dynamics of the increase in erythroid differentiation enchanced in the case of the 5mM treatment.

- A microarray experiment was performed on K562 cells following IP6 administration: 750 μM for shorter times (30 and 60 min) and 5 mM for longer time (12 hrs). We detected non-characteristic changes in gene expressions as a result of a nonspecific shock after administration of a concentrated chemical agent at 30 minutes (phase of adaptation). Significant effects of IP6 started to appear after 1 hour, and after 12 hours. The expression of a subset of genes representing inflammation profile and reduction in cell proliferation are described.
- The 750 μM IP6 for 60 min treatment significantly affected total 1818 transcripts, 771 of which was upregulated and 1041 downregulated. As a result of the treatment with 5 mM IP6 for 12 hrs, expression of 1243 transcripts was changed significantly (615 genes upregulated and 628 genes downregulated). Microarray data were consistently confirmed using real-time PCR of 10 previosly selected genes.
- In comparison with non-treated K562 cells expression of the MYC protooncogene decreased. Higher dose of IP6 represented a more enchanced downregulation. Similar tendency could be observed at TERT transcription, where mRNA level of the enzyme showed gradual gradual decrease with time and dose. Expression level of H-RAS and K-RAS also decreased after IP6-treatment in a dose-dependent manner. The quantity of p21CIP1/WAF1 transcript, a relevant tumor suppressor grew with time upon IP6 administration. This increase was much more pronounced at a higher (5 mM) dose of the drug. The ratio of BAX (proapoptotic) and BCL2 (antiapoptotic) transcript clearly indicates the activation state of apoptosis. In our experiments BAX expression gradually overwhelmed that of BCL2, howewer, after a culmination at 6 hrs a decrease in the ratio could be observed. In agreement with this, the mRNA-level of TP53, another key molecule in apoptosis shows mild increase upon treatment with 750 μM IP6, especially at the ealy hours. The higher IP6 dose, in contrast, led to the decrease of the TP53 transcript. Regarding the two main transcipts of BCR-ABL fusion gene of K562 cells (b2a2, b3a2) we observed unequivocal decrease in quantity of both transcripts at the higher concentration (5 mM) of IP6.

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- A single treatment with Ribavirin resulted in a marked growth inhibition of breast (MCF-7) and ovarian cancer (OVCAR-5), hepatocellular carcinoma (HepG2) and

leukemia (K562) cells. Ribavirin activated apoptosis as well as erythroid differentiation in a dose- and time-dependent manner in leukemia cells.

- cDNA microarray analysis revealed a marked early effect on gene expression modulating 2679 known genes (at $p<0.001$) with a significant difference of expression between the Ribavirin treated and the untreated K562 cells. Early (1 hr) changes of gene expression were observed: 1516 of these genes showed unregulated signals and 1163 down modulation. Ribavirin at low dose (15 μM) had very little effect on gene expression after 12 hrs treatment; whereas treatment with 195 μM resulted in major changes in gene expression (866 genes were up- and 830 down-modulated). Significantly over-represented groups of genes were seen on the oxidative phosphorylation pathway, ribosome assembly and amino acid metabolism. Among additional affected pathways are apoptosis, integrin mediated adhesion, proteasome, histone assembly and chemokine family. Genes containing RNA and GTP-binding domains are significantly involved in the cellular response to the treatment.
- Furthermore, 96 genes were selected with relevance to the major pathways that were expected to be responsible for the effects of Ribavirin (50 μM, up to 48 h) on apoptosis, differentiation, translation initiation and guanylate metabolism. This selected group also included oncogenes, tumor suppressors and other factors belonging to main cancer-related signaling pathways. In response to treatment with 50 μM of drug, pro-apoptotic factors showed either down-regulation (BAX, BID, BIRC5, FAD, TNFRSF1A, FAS) or up-regulation (BAD, BBC3, PMAPI) of gene expression at all tested time points. Several genes belonging to the same group showed an early (3 hrs) downregulation, followed by a late (48 hrs) upregulation (Caspase genes, NFKB2 and NFKBIA). The majority of the differentiation-linked genes tested (HBB, Globin A, MPO) showed increased expression upon Ribavirin treatment, whereas AKT1 was down-regulated until 48 hours using 50 μM Ribavirin. Most genes of the purine metabolism and translation initiation were down-regulated (IMPDH II, PARP1, EIF2A, EIF2B1 and EIF4G1). The mRNA expression of the selected oncogenes (ABL, CCND1, CCNE1, CDKN1B, HRAS, MDM2, MYC, PAK1, TERT) were also down-regulated whereas the tumor suppressors RB1, TP53 and TSC2 showed elevated mRNA levels up to 48 hours using 50 μM of the drug. Treatment with 15 μM Ribavirin also resulted in a significant

decrease of C-MYC, TERT, b3a2 transcript and ABL expression while the level of CIP1/WAF1 was increased for 12 hours.

CONCLUSIONS

The results of the cellular and molecular analyses demonstrate that

- a complex series of molecular events prepares Ribavirin-treated cells for the inhibition of proliferation, the induction of programmed cell death and/or initiation of differentiation.
- the antiproliferative and cytotoxic effect of IP6 was noticed as a result of the treatment of K562 cells compared to the non-treated cells in a time- and dose-dependent manner. The most severe decline in cell survival was detected in the case of the higher, pharmacological concentration (5 mM IP6).
- IP6 is a strong inducer of differentiation and a moderately strong inducer of apoptosis.
- Ribavirin treatment of K562 cells resulted in a major decrease of the IMPDH II mRNA level, which is the predominantly expressed IMPDH isoform in cancer cells.
- Ribavirin treatment decreased the expression of eIF4G, eIF2A and eIF2B genes in K562 cells. These results strongly support the recently discovered inhibitory role of Ribavirin on translation initiation.
- Ribavirin or IP6 also caused a significant decrease in the expression of the major BCR-ABL mRNA variant (b3a2), which may open up new opportunities towards a specific drug combination for the therapy of leukemia.

Our results provide new insights into the signaling events and gene expression changes associated with growth inhibition and apoptosis/differentiation in leukemia cells and may suggest that either the Ribavirin or the IP6 by itself should be effective against various tumor cells.

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