Pharmacokinetics and pharmacogenetics of high-dose methotrexate treatments in pediatric acute lymphoblastic leukemia

Ph.D. thesis

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Introduction

Pediatric acute lymphoblastic leukemia (ALL) accounts for one third of childhood malignancies. The 5-year event-free survival rates (EFS) for ALL now range between 76-86% in children receiving protocol-based therapy in the developed countries. Chemotherapeutics used in the treatment of ALL may result in unfavourable toxicities. Dosing based on body surface area or weight, which is used in the routine clinical practice, might be inappropriate even after personal modification in case of organ damage. Therefore therapeutic drug monitoring of cancer chemotherapy has a great importance, and extensive knowledge of the pharmacokinetics of the different anticancer agents is required.

The classification and treatment of pediatric ALL in Hungary are based on international BFM protocols. Three risk groups (standard, intermediate and high risk groups) were defined based on the age of the patient, white blood cell count, early treatment response, genetic markers according to the ALL-BFM 95 and ALL IC-BFM 2002 protocols that were used in Hungary between 1995 and 2011. Recently the ALL IC-BFM 2009 protocol is applied, in which hypodiplody and the presence of minimal residual disease after induction therapy pertain to the prognostic factors. The treatment of ALL consists of induction, consolidation and maintenance therapies with central nervous system (CNS) prophylaxis. High-dose methotrexate (HD-MTX) is administered in the doses of 2 g/m^2 or 5 g/m^2 as 24 hours long continuous intravenous infusions during the consolidation phase of chemotherapy. HD-MTX infusions are followed by calcium folinate rescue to reduce toxicity.

MTX is an antifolate cytostatic agent. After entering the cells it inhibits the production of cofactors that are necessary for the synthesis of thymidylate and purines. The lack of essential nucleotides leads to the apoptosis of cancer cells with high proliferation rate. MTX enters the cell with active transport mechanisms. Inside the cell MTX is converted to its active polyglutamate form (MTXPG), which inhibits the enzymes dihydrofolate reductase (DHFR), thymidylate synthetase (TYMS) and ribonucleotide transformylases. This latter action might responsible for its immunosuppressive effect. MTX has been widely used as an anticancer agent for a long time. Despite our experience with the drug, recent treatment protocols vary markedly in terms of the timing, dosing and schedule of MTX. Defining the optimal MTX dose, predicting the efficacy and toxicity of HD-MTX treatments are still the aims of numerous studies. Nephrotoxicity, hepatotoxicity, myelotoxicity, gastrointestinal mucositis and neurotoxicity are the most common acute side effects following high-dose treatments. According to previous studies, higher serum MTX levels or delayed elimination have been

shown to correlate with acute toxicity, however there are other studies that do not confirm these associations. The major metabolite of the drug, 7-hydroxy-methotrexate (7-OH-MTX), contributes toward the MTX activity and toxicity.

The pharmacokinetics and pharmacodynamics of the drug show large interpatient variability even with the same treatment protocol. This diversity, the increased MTX sensitivity or resistance, can be partly explained by genomic alterations (mainly single nucleotide polymorphisms, SNPs) in genes involved in the MTX metabolism, cellular transport and effector targets or target pathways.

Hepatic uptake of MTX involves the solute carrier organic anion transporters family, members 1B1 and 1B3 (SLCO1B1 and SLCO1B3). The product of the *SLCO1B1* gene is localized at the sinusoidal membrane of hepatocytes and enterocytes. Polymorphisms of the *SLCO1B1* gene might alter the transporter function. The most extensively studied non synonym polymorphism, the rs4149056 (521T>C, Ala174Val), results in decreased transporter activity regarding several substrates. The association between this SNP and the pharmacokinetics of MTX was confirmed previously.

The AT rich interactive domain 5B gene (*ARID5B*) encodes a member of the ARID family of transcription factors. The *ARID5B* gene plays roles in the adipogenesis and liver development, but also in cell growth and differentiation of B-lymphocyte progenitors. SNPs of this gene have previously been associated with higher susceptibility to ALL and B-hyperdiploid subtype of ALL. These genetic variations of *ARID5B* have also been linked to greater MTXPGs accumulation in the B-hyperdiploid ALL patients. It is known that B-hyperdiploid ALL has a better response to HD-MTX therapy. Together, these findings suggest that leukemogenesis and antileukemic drug response may converge on common – up to this point undefined - pathways.

Objectives

During my work I pursued the following objectives:

Pharmacokinetic studies:

1. To analyze and compare the pharmacokinetic parameters of the HD-MTX treatments used in Hungary regarding MTX and 7-OH-MTX in both serum and cerebrospinal fuid (CSF).

2. Investigate and compare the development of acute toxicity after administering various doses of HD-MTX infusions.
3. Investigate the differences in the pharmacokinetics and toxicity of HD-MTX according to age and gender.

Pharmacogenetic studies:

4. To investigate the influence of SNPs in genes of the folate metabolic pathway, transporter molecules and transcription proteins on the pharmacokinetics of MTX and 7-OH-MTX.
5. To investigate whether these SNPs are associated with the development of acute toxicity after HD-MTX treatment.

Methods

The data of 153 patients diagnosed with ALL between 1998 and 2011 in the 2nd Department of Pediatrics of Semmelweis University were analyzed. All patients who received the HD-MTX treatment were included. The data were collected by retrospective chart review. The patients were treated according to the ALL-BFM 95 and ALL IC-BFM 2002 protocols. A total of 241 cycles (65 patients) of MTX at 5 g/m^2/24 hours and 342 cycles (88 patients) of MTX at 2 g/m^2/24 hours were analyzed and compared. During our pharmacokinetic studies the patients were divided into subgroups according to age: children younger than 6 years of age (<6 years) and children older than 14 years of age (>14 years) at diagnosis. We investigated the pharmacokinetics and toxicity in these groups of children.

DNA samples could be obtained from 118 patients. For this reason MTX cycles of these patients only were included in our pharmacogenetic studies. Patients who passed away before the collection of the DNA samples were not included.

For the pharmacokinetic analyses mean and median values of the measured serum MTX and 7-OH-MTX levels at 24, 36 and 48 hours were used. We investigated the simultaneous alteration of the MTX or 7-OH-MTX levels (at 24h + 36h + 48h – as one multivariate response variable) and we calculated the area under the concentration-time curve (AUC) of MTX and 7-OH-MTX as well. The CSF MTX penetration ratio was estimated by dividing the CSF MTX level at 24 hours by the serum MTX levels at 24 hours. The CSF MTX levels were measured immediately before the intrathecal MTX prophylaxis. The serum hemoglobin levels, white blood cell counts, granulocyte counts, platelet counts, total protein

levels, transaminase levels (ALT and GGT), bilirubin levels and creatinine levels were collected from the blood samples on days 1, 2 and 7 after each HD-MTX infusion. Toxicity values were evaluated by applying the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 scoring system. The associations between the presence of the most severe (grade3-4) toxicity and the pharmacokinetics of MTX or 7-OH-MTX and the selected genetic variants were analyzed.

We selected 63 polymorphisms of 14 candidate genes based on the literature for our pharmacogenetic analyses. Genomic DNA from the children was obtained retrospectively from whole peripheral blood samples taken in remission using QIAmp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping was carried out by Sequenom iPLEX Gold MassARRAY technology at the McGill University and Génome Québec Innovation Centre, Montréal, Canada.

The pharmacokinetic and toxicity analyses were conducted with the StatSoft STATISTICA (version 7.0) program. Parametric (Student *t*-test, Pearson's correlation) and nonparametric (Mann-Whitney U, chi-square and Wilcoxon signed-rank tests, and the Spearman correlation) tests were used according to the distributions of the variables. During the pharmacogenetic analyses Hardy-Weinberg equilibrium was tested by using a chi-square goodness of fit. A SNP was excluded from the statistical analysis if the frequencies of the wild type and variant alleles deviated from the Hardy-Weinberg equilibrium requirement (a total of 4 SNPs were excluded). The relationships between the SNPs and the pharmackokinetic or toxicity parameters were analyzed by the R program (version 2.15). In the first step, random forest method was applied to calculate variable importance measures because of the large number of explanatory variables and the relatively small sample size. In the second step, we built classification and regression trees (CART) with the selected explanatory variables to generate clinical decision rules and to explore the relationship between the response and explanatory variables. In the last step, the linear mixed models were applied to prove the significance of the selected variables and their interactions by the CART and to estimate effect sizes. The linear mixed models enabled the correlated nature of the data to be taken into account.

Results

The MTX and 7-OH-MTX serum levels at 24, 36 and 48 hours were significantly higher after 5 g/m^2/24 hours of HD-MTX (in the MTX5 treatment group, $p<0.0001$).

Considerable interindividual and intraindividual differences were observed in both groups. The AUC_{MTX} and $AUC_{7\text{-OH-MTX}}$ values calculated were significantly higher in the MTX5 treatment group ($p<0.0001$).

The suggested therapeutic level (10-100 μmol/l at 24 hours) was achieved in significantly fewer cases after 2 g/m^2/24 hours of HD-MTX (MTX2 treatment group). The serum levels of MTX at 24 hours were less than 10 μmol/l in 9.88% of the MTX2 cases and in 1.67% of the MTX5 cases ($p=0.0007$). In the MTX5 treatment group, the serum levels of MTX at 24 hours mostly ranged from 30 to 100 μmol/l, but this range was achieved in significantly fewer MTX2 cases (MTX5: 76.15% vs. MTX2: 32.33%, $p<0.0001$). A 100 μmol/l concentration was exceeded in 17.57% of the MTX5 cases and in 1.19% of the MTX2 cases ($p<0.0001$).

No differences in either group were found when the MTX and 7-OH-MTX levels after 1-4 cycles were compared. The AUC_{MTX} and $AUC_{7\text{-OH-MTX}}$ values were similar in both groups in cycles 1-4. The CSF level of MTX also remained unchanged.

The CSF MTX concentration was significantly higher in the MTX5 group ($p<0.0001$). An analysis of the distribution of the CSF levels indicated that the MTX concentration mostly ranged from 1 to 10 μmol/l in the MTX5 treatment group (MTX5: 63.92% vs. MTX2: 22.72%, $p<0.0001$) but was below the cytotoxic 1 μmol/l threshold in most of the MTX2 cases (73.33%). A 10 μmol/l concentration was exceeded in 8.25% of the MTX5 cases and in 3.92% of the MTX2 cases.

There was no significant difference in the CSF MTX penetration ratio between the groups. The median values of the CSF MTX_{24h}/serum MTX_{24h} ratios were similar in both groups: MTX5: 2.3% (95% CI: 1.7-2.5%) vs. MTX2: 2.8% (95% CI: 2.4-3%). The proportion of CSF penetration did not depend on the infusion dose, which was confirmed by analyzing the correlation between the serum MTX, serum 7-OH-MTX and CSF MTX concentration. The serum MTX level at 24 hours and CSF MTX values showed a significant correlation in both groups (MTX5: Spearman $r=0.36$, $p=0.0002$; MTX2: Spearman $r=0.38$, $p<0.0001$).

Most of the developed toxicities were mild (< grade 3). Hepatotoxicity (grades 3-4) occurred in significantly more MTX5 cases on days 1-7 after the HD-MTX infusion (MTX5: 12.4% vs. MTX2: 5.3%, $p=0.006$). Leukocytopenia and granulocytopenia (grades 3-4) were found in significantly more MTX5 cases on day 7 (MTX5: 36.9% vs. MTX2: 15.5%, $p<0.0001$). On days 1-2, the total serum protein decreased (< 60 g/l) in approximately half of the cases in each group: MTX5: 49.4% vs. MTX2: 59.4%, $p=0.08$. Toxic creatinine levels (grades 3-4) were found on days 1-7 in more MTX5 than MTX2 cases, but no significant difference was found between the groups: MTX5: 3.2% vs. MTX2: 1.9%, $p=0.50$.

The 7-OH-MTX levels at 24 hours in the MTX5 group showed a correlation with the peak creatinine levels on days 1-2 (Pearson r=0.42, p<0.0001) and on day 7 (Pearson r=0.36, p<0.0001) after the HD-MTX infusions. The $AUC_{7\text{-OH-MTX}}$ values were correlated with the peak creatinine levels on days 1-2 in the MTX5 treatment group (Pearson r=0.41, p<0.001) and with the creatinine levels on day 7 in both groups (MTX5: Pearson r=0.34. p=0.001; MTX2: Pearson r=0.29, p=0.016). Neither hepatotoxicity nor bone marrow toxicity was correlated with the 7-OH-MTX levels in either group.

For those cases in which the increases in creatinine level were greater than 20 μmol/l on day 7 compared with day 0, higher 7-OH-MTX concentrations were found at 24 hours, but the difference was not significant (p=0.07 in the MTX5 treatment group).

There was no linear (Pearson's) correlation between the MTX pharmacokinetics and toxicity in either group. However, in the MTX2 cases with hepatotoxicity on days 1-7, the mean AUC_{MTX} values were significantly higher compared with MTX2 cases with no hepatotoxicity (p=0.005).

In both treatment groups children older than 14 years of age had a significantly higher MTX concentration at 48 hours than children younger than 6 years of age (MTX5: p=0.0013, MTX2: p=0.0021). In the children aged >14 years in the MTX5 treatment group, we found significantly more cases of toxic creatinine levels on days 1-2 (>14 y: 12% vs. <6 y: 0.8%, p=0.019) and on day 7 (>14 y: 16.7% vs. <6 y: 2.1%, p=0.014). Hepatotoxicity on days 1-2 was found in significantly more cases among children >14 years in the MTX2 treatment group (>14 y: 8.7% vs. <6 y: 0.5%, p=0.024).

No difference between the genders in the MTX5 treatment group was found regarding pharmacokinetics of MTX or 7-OH-MTX. The MTX levels at 24 hours and the AUC_{MTX} values were significantly higher in girls in the MTX2 treatment group (p=0.001 and p=0.002).

In our pharmacogenetic analyses the CART of the serum MTX levels (24h + 36h + 48h) showed that the administered MTX dose (5 or 2 g/m^2) had the strongest effect on the serum MTX levels (p<0.001). In patients who received 2 g/m^2, *ARID5B* rs4948496 showed a significant association with the serum MTX levels (p=0.01). Patients with the CC genotype were found to have higher serum MTX levels than in patients with the CT+TT genotype. In patients who received 2 g/m^2 MTX and had the *ARID5B* rs4948496 CT+TT genotype, the *SLCO1B1* rs4149056 also showed a significant association with the serum MTX levels (p<0.001). Patients with the TT genotype had lower serum MTX levels than patients with CT+CC genotype. In patients who received 2 g/m^2 MTX associations between both SNPs and serum MTX levels were confirmed with the general linear mixed models (GLMM)

(rs4948496: p=0.039, rs4149056: p=0.004). In patients who received 2 g/m^2 MTX and had the CC genotype of the *ARID5B* rs4948496 high (>0.25 μmol/l) MTX levels at 48 hours were found more frequently: CC vs. CT+TT genotype: 61% vs. 39%, p=0.044.

The CART of the serum 7-OH-MTX levels (24h + 36h + 48h) showed similar results to the CART of the serum MTX levels. The administered MTX dose (5 or 2 g/m^2) had the strongest effect on the serum 7-OH-MTX levels (p<0.001). In both groups the *ARID5B* rs4948502 showed significant associations with the serum levels (5 and 2 g/m^2: p=0.015 and p<0.001), which was also confirmed by the GLMM (5 and 2 g/m^2: p=0.003 and p=0.033). However, the associations between the SNP and serum levels in the two groups are not unambiguous. Higher serum 7-OH-MTX levels were found in patients who received 2 g/m^2 MTX and had the *ARID5B* rs4948502 CC genotype, whereas in patients who received 5 g/m^2 MTX the CC genotype was associated with lower serum 7-OH-MTX levels (in comparison with patients with the CT+TT genotype).

The result of the CART of the calculated $AUC_{7\text{-OH-MTX}}$ was similar to the CART of the serum 7-OH-MTX levels. The *ARID5B* rs4948502 showed a significant association with the AUC of 7-OH-MTX in both groups (5 and 2 g/m^2: p<0.001 separately), which was confirmed by the GLMM (5 and 2 g/m^2: p<0.001 and p=0.013).

The CART showed significant associations between hepatotoxicity and the *SLC19A1* rs7499 (p=0.003) and *ARID5B* rs4948502 (p=0.007) in patients who received 5 g/m^2 MTX.

Nephrotoxicity was associated with the *SLC22A8* rs4149183 in patients who received 5 g/m^2 MTX. Nephrotoxicity was found significantly more frequently in patients with CC genotype than in CT+TT genotype (25% vs. 1%, p=0.001).

Hypoproteinaemia was associated with the *ARID5B* rs4948487 (p=0.004) and the *MTHFD1* rs1076991 (p<0.001) in patients who received 2 g/m^2 MTX. Hypoproteinaemia occurred more frequently in patients with the *ARID5B* rs4948487 AA genotype and in patients with the *MTHFD1* rs1076991 GG genotype.

The CART and the generalized linear mixed model (GzLMM) showed a significant association between the granulocytopenia and the *MTR* rs3768142 in patients who received 2 g/m^2 MTX [GG vs. GT+TT genotype: 56% vs. 23%, OR: 5.92 (95% CI: 2.03-17.27), p=0.001]. Granulocytopenia occurred significantly more frequently after the last (4th) MTX cycle than after the others (cycles 1-3); OR: 3.48 (95% CI: 1.40-8.64), p=0.007.

Conclusions

Our data comparing the 5 and 2 g/m^2 treatments showed that therapeutic serum MTX levels can be achieved more reliably with higher dose infusions, although the higher dose also has slightly more, but reversible side effects. Repeated treatment cycles do not alter the serum levels or AUC of MTX and 7-OH-MTX and do not cause more toxicity, consistent with the results from previous studies on leukemia patients. Data from our and previous studies show that higher MTX levels and toxicity occur significantly more frequently in adolescents (>14 years) than in patients younger than 6 years of age.

The serum and CSF MTX levels showed great interindividual and intraindividual differences after the 5 g/m^2 and 2 g/m^2 treatments. The correlation between the serum and CSF MTX levels are inconsistent in the literature. We examined whether the CSF penetration differs depending on the infusion dose. Our results showed that the CSF penetration rate is dose independent, and is approximately equal after the 5 and 2 g/m^2 infusions. The MTX levels at 24 hours in the serum and CSF were also found to be significantly correlated.

We found a strong relationship between the serum levels of 7-OH-MTX and the developed nephrotoxicity in both treatment groups. It is recommended to investigate whether the serum levels of 7-OH-MTX show closer correlation with the development of acute toxicity after HD-MTX, hence monitoring of the metabolite may be more beneficial.

In our pharmacogenetic analyses we applied relatively new multivariate analyses which are well-suited when a small sample size with large number of observations is given. We could test the potential interactions of the variables as well.

This is the first study that found significant associations between the polymorphisms of the *ARID5B* gene and the pharmacokinetics and toxicity of MTX. However, the exact role of this gene on MTX levels and toxicity needs further investigations.

Our results confirm the studies that suggest *SLCO1B1* is a novel predictor for MTX clearance and toxicity.

Novel genetic variants of the *SLC19A1*, *SLC22A8*, *MTR* and *MTHFD1* genes were shown to be associated with the development of acute toxicity after HD-MTX treatment.

After further investigations and validation in a larger cohort, our pharmacokinetic and pharmacogenetic results might contribute to the individual dose adjustment.

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