Steady-state of azathioprine during initiation treatment of pediatric inflammatory bowel disease

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Received 28 March 2010; received in revised form 20 June 2010; accepted 21 June 2010

KEYWORDS
Inflammatory bowel diseases; Children; Azathioprine; Steady state

Abstract

Background and Aim: Azathioprine (AZA) has a slow onset of action in treatment of pediatric inflammatory bowel disease (IBD). It is anticipated, that this delay correlates to the kinetics of 6-thioguanine nucletiodes (6-TGN) accumulation. The aim of this study was to evaluate the time to steady state of 6-TGN concentration in red blood cells.

Methods: The inclusion criteria were: a) age 0-19 years b) IBD diagnosis c) AZA treatment initiation. High performance liquid chromatography was used for the 6-TGN analysis. Concentrations of metabolites were studied in weeks 0, 1, 2, 5, and 8 after beginning of treatment.

Results: The inclusion criteria were matched to 18 patients with IBD. The median time to steady state of 6-TGN was 55.3 days. The mean 6-TGN concentration at the steady state achieved 326 (SD 154) pmol/8.108 erythrocytes. High erythrocyte TPMT activity corresponds to the low steady state 6-TGN concentration and vice versa. This correlation reached statistical significance (p<0.01) for the dose expressed in mg per square meter of body surface area.

Conclusion: The time to steady state of 6-TGN erythrocyte concentration is significantly shorter than would expected according to clinical observation describe earlier.

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1. Introduction

Inflammatory bowel diseases (IBD) are diagnosed in 20–30% of patients during their childhood and adolescence. Systemic steroids are frequently used during the acute phase of the disease, but they are usually quickly replaced by maintenance treatment with thiopurine derivates — azathioprine (AZA) or 6-mercaptopurine (6-MP). AZA is an ineffective pro-drug with better reabsorption from gastrointestinal tract (GIT) than 6-MP. Approximately 90% of the absorbed AZA undergoes a rapid conversion to 6-MP, which is then metabolised by three competitive pathways. Thiopurine

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methyltransferase (TPMT) and xanthinoxidase are key enzymes of two catabolic pathways producing 6-methylmercaptopurine nucleotide (6-MMPN) and 6-thiouric acid, respectively. The desirable metabolic formation is catalysed by hypoxanthine phosphoribosyltransferase and the final active substance represents 6-thioguanine nucleotides (6-TGN). The main mechanism of the action resides in the cumulation of 6-TGN and their incorporation into cells’ deoxynucleic acid. The synthesis of DNA, RNA, and proteins de novo is altered and the products of such a synthesis are defective. The lymphocyte apoptosis induction by 6-thioguaninetriphosphate nucleotides contributes to the clinical effect of 6-thiopurine derivates.\(^1\)

The aim of this study was an investigation of AZA pharmacokinetics after the pediatric IBD treatment initiation, including the 6-TGN and 6-MMPN time to steady state analysis.

2. Materials and methods

2.1. Study patients

The trial design was an open prospective single centre study from April 2007 to April 2009. All the inclusion criteria of the study had to be matched.

1) Crohn disease (CD) or ulcerative colitis (UC) diagnosed according to Porto criteria.\(^2\)
2) Age 0–19 years.
3) Azathioprine treatment initiation.

Blood samples were taken during routine monitoring of azathioprine potential side effects.\(^3\) There were no other indications for blood drawings. The first sample was taken in time 0, before beginning of AZA treatment, and then followed by samples in week 1, 2, 5, and 8. The TPMT activity and genotype analysis were performed according to Porto criteria.\(^2\)

The TPMT activity and genotype analysis were performed in time 0.6-TGN and 6-MMPN concentrations were analyzed in week 0, 1, 2, 5, and 8. Results of genotype, TPMT activity, and metabolite concentrations were not available whilst the study was running — therefore no interventions (e.g. dose changes) were possible.

2.2. Disease activity, remission, partial therapeutic remission

Pediatric Crohn’s disease activity index (PCDAI) was used for the monitoring of therapeutic response and disease severity.\(^3\) PCDAI ≤30 points was a marker of moderate to severe disease activity, mild disease activity was defined as PCDAI between 11 and 30 points. PCDAI ≤10 points was used as a criterion of remission. Partial therapeutic response was defined as a decrease of PCDAI about 12.5 or more points, but remission was not achieved. Disease activity of UC was defined as a decrease of PCDAI about 12.5 or more points, criterion of remission. Partial therapeutic response was defined as a decrease of PCDAI about 25 or more points.

2.3. The TPMT activity determination

The TPMT activity in red blood cells (RBC) was evaluated as described elsewhere\(^6\) with the following minor modifications. Volume 100 µL of RBC was treated with 20 µL 6 µM 6-mercaptopurine and 20 µL 1.5 µM S-adenosylmethionine for 90 min at 37 °C. Enzymatically formed 6-methylmercaptopurine was measured in three aliquots taken at 30, 60, 90 min of the incubation. The activity was determined from the slope of concentration vs. time curve.

2.4. Thioguanine, mercaptopurine and methylmercaptopurine HPLC assay

The assay was similar to that published previously.\(^6\) The sample of 200 µL RBC was mixed with 25 µL of dithiothreitol (c=0.5 M) and deproteinized with perchloric acid of volume 50 µL. The supernatant was incubated for 90 min at 98 °C and analysed. The separation was carried out by the gradient method on a reversed-phase column MERCK Purospher STAR RP-18e (250× 4 mm, 5 µm). The flow rate was 1.0 ml/min. The mobile phase A consisted of sodium dihydrogen phosphate (100 mM, pH=2.7), the mobile phase B consisted of sodium dihydrogen phosphate (100 mM, pH=3.5) mixed with methanol in 1:1 volume ratio.

2.5. TPMT genotyping

TPMT genotyping (*1 wild-type, *2, *3A, *3B, and *3C alleles) was performed on DNA samples extracted from peripheral blood cells. Each reaction was composed from 2 µl of LightCycler FastStart DNA Master Hybridization Probes, 3 µmol/l MgCl, 1 µl of LightSNiP Reagent Mix, and 50 ng 2 of genomic DNA. The alleles *3A, *3B and *3C were examined using an initial denaturation 95 °C (10 min) and 50 cycles of 95 °C (10 s), 60 °C (10 s), 72 °C (15 s). After the amplification, melting analysis was performed by denaturation at 95 °C (30 s), annealing at 40 °C (2 min), and slow heating to 75 °C with a ramp rate of 0.2 °C/s.

2.6. Pharmacokinetics and statistical analysis

The time to steady state of 6-MP metabolites in each subject was estimated using compartmental pharmacokinetic modelling in the GraphPad Prism 5.0 software (San Diego, CA, USA). Due to the relatively low numbers of concentration data, the one-compartment model was used and no other models were tested.

Time to steady state was estimated according to the equation $t_{90\%} = \ln(0.1/(1 - K))$, where $K$ is the equilibration velocity constant, which corresponds to the rate constant of the elimination of the one-compartment model. The activity of TPMT and concentrations of 6-MP metabolites were logarithmically transformed before the statistical analysis with parametric tests. Relationships between the dose of AZA, TPMT activity and 6-TGN steady state concentration were assessed using Pearson’s coefficient of correlation.
2.7. Ethical consent

The protocol of this prospective open single centre study was approved by the local ethics committee. The attendance at the study was voluntary; informed consent was obtained from the patients or their parents.

3. Results

3.1. Patients

The main characteristics of the 18 children included are summarised in Table 1.

Fifteen children (7 females) had CD. Two of them had ileal disease, 8 ileocolonic, 2 colonic disease, and in 3 patients the disease was limited to the proximal small bowel and/or upper gastrointestinal tract. Two patients had coexistent associated perianal fistulas. Ulcerative colitis was diagnosed in 3 patients (2 females).

The indication for azathioprine (Imuran, GlaxoSmithKline Pharmaceuticals S.A.) treatment was cortico-dependent CD (n = 14), maintenance therapy after surgically achieved remission of CD (n = 1) or maintaining of conservatively achieved remission of UC (n = 3).

The median of the study duration was 154 days (64–266 days), the mean initial AZA dose was 1.45 (SD 0.36) mg/kg of body weight (Table 2) in one daily dose. Azathioprine therapy was not discontinued during the study, the concomitant medication is depicted in Table 1.

3.2. Primary outcomes

Results and the statistical evaluation of the equilibration velocity constant, the half time of steady state equation, the time to steady state and balanced concentrations of 6-TGN and 6-MMPN in erythrocytes are presented in Table 2.

The median time to steady state of 6-TGN was 55.3 days, the mean 6-TGN concentration at the steady state achieved 326 (SD 154) pmol/8 × 10^10 erythrocytes.

We observed inconclusive correlations between the erythrocyte 6-TGN steady state concentration and AZA dose per kilogram of body weight or square meter of body surface area (Fig. 1).

The high erythrocyte TPMT activity corresponds to the low steady state 6-TGN concentration and vice versa. This correlation reached the statistical significance (p < 0.01) for the dose expressed in mg per square metre of body surface area (Fig. 2).

When the dose in mg per kilogram of body weight was used in the above relationship, the correlation was only of borderline significance (p = 0.054). Considerable intra-individual fluctuations of erythrocyte 6-MMPN levels were frequent. An acceptable agreement between measured concentrations and those predicted by the one-compartment model was observed in 6 patients only (Table 2).

The remission was achieved in 11 and partial therapeutic response was achieved in 3 children with CD at steady state time. The remission in one patient (No. 14) was achieved surgically and the indication for AZA treatment was maintenance therapy.

Moderate or mild activity was observed in 3 children with UC at steady state time (Table 2).

4. Discussion

Azathioprine has been used for the treatment of inflammatory bowel disease for more than 30 years and is generally accepted as a safe and effective drug. Markowitz and co-workers proved in a well-designed and quite large cohort that the therapy with 6-mercaptopurine leads to lower exposition to corticosteroids and lower frequency of corticosteroid dependency. Although the results of this paper are well known, the approach to newly diagnosed children with inflammatory bowel disease is far from unique. In two recent retrospective multicentric studies published, azathioprine was used only in 2–5% of newly diagnosed children with Crohn disease during the first month of treatment.

A different approach is known from the USA, where 20% of American pediatric gastroenterologists supposed Crohn disease diagnosis enough for the indication of an immune modulator treatment. An international survey found that 45% of pediatric gastroenterologists from Western Europe, USA and Israel advocate azathioprine or 6-mercaptopurine treatment when the active disease requires steroids application and 93% of them would use this medication for relapsing disease.

AZA has a slow onset of action, with mean time to response of 3 month. Hyams JS stated that maximum efficacy can be expected in 12–24 weeks in children. It is anticipated that this delay correlates to 6-TGN accumulation and steady state achieving. The aim of the presented paper resides in the pharmacokinetic study and the precise description of the above mentioned observation. The mean time to steady state of 6-TGN erythrocyte concentration observed in this study was 55 days (median 55 days). This interval (approx. 8 weeks) is notably shorter than described by Hyams. The shortest time to steady state — only 2 weeks — was observed in two patients (No. 2, 16) diagnosed as CD. In adults with Crohn disease, Hindorf U et al. found a mean time to steady state of 6-TGN 5 weeks. It seems that in an important number of patients the time to steady state is significantly shorter than expected and therefore also the therapeutic effect of azathioprine can be expected sooner in most children.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics and concomitant medication.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn disease</td>
<td>n = 15</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>n = 3</td>
</tr>
<tr>
<td>Mean of age (median)</td>
<td>14,8 (15,5) year</td>
</tr>
<tr>
<td>Minimum of age</td>
<td>5,1 year</td>
</tr>
<tr>
<td>Maximum of age</td>
<td>18,4 year</td>
</tr>
<tr>
<td>Girls</td>
<td>n = 9</td>
</tr>
<tr>
<td>Boys</td>
<td>n = 9</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>n = 16</td>
</tr>
<tr>
<td>Budesonide</td>
<td>n = 1</td>
</tr>
<tr>
<td>5-ASA</td>
<td>n = 15</td>
</tr>
<tr>
<td>Chinoline/metronidazol.</td>
<td>n = 6</td>
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### Table 2  Genotype and phenotype of thiopurine methyltransferase (TPMT) and pharmacokinetic characteristics of 6-TGN and 6-MMPN accumulation in erythrocytes of children under study.

<table>
<thead>
<tr>
<th>ID</th>
<th>Diagnosis</th>
<th>Dose mg/kg b.w.</th>
<th>TPMT Genotype</th>
<th>TPMT Activity</th>
<th>Clinical outcome</th>
<th>6-TGN</th>
<th>6-MMPN</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(K)</td>
<td>(t_{90%})</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>((\text{day}^{-1}))</td>
<td>((\text{day}))</td>
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<td>1</td>
<td>CD</td>
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<td>*1/*1</td>
<td>38.69</td>
<td>Remission</td>
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<td>36.8</td>
</tr>
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<td>2</td>
<td>CD</td>
<td>1.34</td>
<td>*3A/*1</td>
<td>14.48</td>
<td>Remission</td>
<td>0.20770</td>
<td>11.1</td>
</tr>
<tr>
<td>3</td>
<td>CD</td>
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<td>*3A/*1</td>
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<td>Remission</td>
<td>0.06562</td>
<td>35.1</td>
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<tr>
<td>4</td>
<td>UC</td>
<td>1.18</td>
<td>*1/*1</td>
<td>44.43</td>
<td>Moderate activity</td>
<td>0.01981</td>
<td>116</td>
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<td>*1/*1</td>
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<td>PTR</td>
<td>0.03061</td>
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<tr>
<td>9</td>
<td>UC</td>
<td>1.57</td>
<td>*1/*1</td>
<td>66.10</td>
<td>Mild activity</td>
<td>0.02544</td>
<td>90.5</td>
</tr>
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<td>CD</td>
<td>0.74</td>
<td>*1/*1</td>
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<td>PTR</td>
<td>0.05547</td>
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<td>11</td>
<td>CD</td>
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<td>38.40</td>
<td>Remission</td>
<td>0.02313</td>
<td>99.5</td>
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<tr>
<td>12</td>
<td>CD</td>
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<td>*1/*1</td>
<td>30.50</td>
<td>Remission</td>
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<td>103</td>
</tr>
<tr>
<td>13</td>
<td>UC</td>
<td>1.54</td>
<td>*3A/*1</td>
<td>12.50</td>
<td>Moderate activity</td>
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<td>47.1</td>
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<td>Remission</td>
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<td>15</td>
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<td>31.84</td>
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<td>Remission</td>
<td>0.15790</td>
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<td>Remission</td>
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<td>25.90</td>
<td>PTR</td>
<td>0.03250</td>
<td>70.8</td>
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<tr>
<td>Mean</td>
<td></td>
<td>1.46</td>
<td>–</td>
<td>32.09</td>
<td></td>
<td>0.0420</td>
<td>54.8</td>
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<tr>
<td>SD</td>
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<td>0.34</td>
<td>–</td>
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<td>0.0219</td>
<td>28.6</td>
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<tr>
<td>Median</td>
<td></td>
<td>1.52</td>
<td>–</td>
<td>30.84</td>
<td></td>
<td>0.0416</td>
<td>55.3</td>
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</table>

ID = patient identification; 6-TGN = 6-thioguanine nucleotides; 6-MMPN = 6-methylmercaptopurine nucleotides; \(C_{ss}\) = steady-state concentration; \(K\) = equilibration velocity constant; \(t_{90\%}\) = time to 90% steady-state equilibration (days) according to formula \(t_{90\%}=\ln(0.1)/(−K)\). TPMT = Thiopurine methyltransferase; CD = Crohn’s disease; UC = ulcerative colitis; PTR = Partial therapeutic response; dose mg/kg b.w. = azathioprine dose mg/kg of body weight; nc = non-concordance between assayed and model-predicted concentrations.
To the best of our knowledge, there are no published studies concerning the time to steady state of 6-TGN erythrocyte concentrations after azathioprine initiation in pediatric IBD. Routine monitoring of 6-TGN from the start of therapy is valuable because it enables an identification of patients with a fast or slow equilibration of effective concentrations. We can argue, in the first group, that the titration of the effective dose can proceed quicker. In the second group, targeting of the therapeutic concentration might be accelerated if higher initial dosing is followed by reduced maintenance dosing of AZA. However, such an approach has no support in the literature and is only speculative until proven otherwise.

Monitoring of azathioprine metabolites levels combined with the TPMT activity may predict a clinical response in patients with IBD. Derijks et al. stated in a review article that the therapeutic target levels of 6-TGN 250–500 pmol/8·10^8 RBC were associated with a higher prevalence of Crohn disease remission.

In this study, the mean erythrocyte 6-TGN concentration at the steady state was 326 (SD 154) pmol/8·10^8 RBC. The clinical response of individual patients in relation to steady state concentrations of 6-TGN is given in Table 2. However, it is necessary to emphasize, that corticosteroids (prednisolone or budesonide) affect the disease activity significantly and play the main role in reaching remission or reaching of partial therapeutic response.

Side effects of azathioprine were not observed, even in the patient with a low activity of TPMT and 6-TGN steady state concentration 1036 pmol/8·10^8 RBC. The dose of azathioprine in this particular patient was reduced immediately after the results were known.

Hindorf and coworkers used, in all adult CD patients, the standard approach with dose escalation to the final dose of 2.5 mg per kilogram of body weight reached in week 3 after the treatment beginning. For the analysis of TPMT-genotype or activity and metabolites measurements are not provided routinely in our institution, the initial azathioprine dose was lower in this study (median 1.5 mg/kg).

The correlation between AZA dose and 6-TGN levels in erythrocytes described by other investigators is weak, presumably due to several factors increasing pharmacokinetic variability of 6-TGN and 6-MMPN, such azathioprine absorption, or drug interactions. Mesalamine, balsalazide, and sulphasalazine all produced increased red blood cell 6-TGN in Crohn’s disease patients in the study of Lowry and colleagues. This report contrasts with the findings of Dubinsky et al., who reported no influence of 6-TNG or 6-MMPN concentration in 48 CD patients with concomitant mesalamine therapy.

In our study we also observed an inconclusive correlation between 6-TGN steady state concentrations and the dose of AZA. Due to a small cohort we cannot assess if the concomitant medication influenced 6-TGN kinetics. As it can be expected, the time to steady state and steady state concentrations differed in 2 patients (No 2, 3) with the TPMT genotype *3A/*1 and the low erythrocyte TPMT activity. In the patient No 3 with this genotype an elevated erythrocyte 6-TGN concentration (1036 pmol/8·10^8 RBC) was found.

Unlike the 6-TGN concentration, the erythrocyte concentration of 6-MMPN of most children was characterised by a large intra-individual fluctuation. The steady state could not

Figure 1  Model-predicted steady-state concentrations of 6-thioguanine nucleotides (6-TGN) in erythrocytes plotted against the daily dose of azathioprine expressed in A: mg/m^2 body surface area, and B: mg/kg of body weight. Open and closed squares are data of homozygotes for allele *1 and heterozygotes for alleles *3A and *1 of thiopurine methyltransferase.

Figure 2  Association between the activity of thiopurine-methyltransferase (TPMT) assayed in erythrocytes before azathioprine treatment initiation and the steady-state concentration of 6-thioguanine nucleotides (6-TGN) in erythrocytes. Dose-normalised concentrations were calculated using the daily dose of azathioprine in mg/m^2 body surface area. Open and closed squares are data of homozygotes for allele *1 and heterozygotes for alleles *3A and *1 of TPMT.
be identified and parameters of the one-compartment model could not be obtained with an adequate precision or the estimation failed. Reasonable fits were obtained only in 6 patients. Abnormal liver tests were recorded in none patient from this group.

The most important finding of this study is the fact that the time to steady state of 6-TGN erythrocyte concentration in children with IBD is significantly shorter than would correspond to the time-course of the clinical effect described earlier. Despite the use of lower initial azathioprine dose than is generally recommended for the treatment of children with IBD, we observed that the steady state concentations of 6-TGN were in the range which several studies saw associated with higher probability of remission. Finally, we suppose that the genotype/phenotype analysis of TPMT and monitoring of 6-TGN levels could shorten the dose finding process after initiation of azathioprine treatment in IBD children.

Acknowledgements

Special acknowledgements to Brian Bolger for the language corrections.

This study was funded in full by IGA of the Ministry of Health of the Czech Republic grant No. NR 9255-3.

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