Methods for Determination of Reactive Metabolites of Thiopurines

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Abstract

Background: This Review Represents A Critical And Constructive Analysis Of Literature In The Content Of Methods For Determination Of Reactive Metabolites Of Thiopurines. The Review Is Generated Through Summary, Classification, Analysis And Comparison Of Already Existing Material And Researches On Field.

Methods: Databases As Scopus, Pubmed, Medline And Web Of Science Were Used To Extract Data For The Review. Search Terms Like "Methods For Determination Of Metabolites", "Thiopurines" And "Reactive Metabolites" Were Used. Out Of 160 Research Articles Screened, 20 Most Relevant Studies Are Included In This Review.

Conclusion: Determining Thiopurine Metabolites Level Is Crucial On Improving Patients' Safety, Quality And Efficacy Of Treatment, Measure Patients' Adherence To Therapy, Optimize Thiopurine Dosage By Either Finding Safer/Higher Doses If Remission Is Not Achieved, Or By Determining Dose Reductions When Adverse Treatment Outcomes Occur.

Thiopurine Metabolites Are Measured By High-Performance Liquid Chromatography (Hplc), Through Determining Metabolites' Concentration In Hemolysate. Thioguanine Nucleotides (Tgn) And The Methylated Thioinosine Derivatives Are Not Measured Directly, Rather The 6-Thioguanine (Tg) For Tgn, And 4-Amino-5-(Methylthio) Carbonyl Imidazole (Amtci) For Methylated Thioinosine Derivatives Are Measured.

There Is A Misconception Among Patients And Healthcare Providers, That Determining Thiopurine Metabolites Level Provides Assurance On Optimal Dosing Which Can Be Used To Prevent Adverse Thiopurine Treatment Outcomes. Despite This Assumption, Clearly Most Of The Thiopurine Adverse Effects Are Not Directly Related To Their Metabolites As 6-Tgn Or 6-Mmp Levels. Clinical Experience Has Demonstrated That Infections Associated With Thiopurines Do Not Regularly Correlate With Leukopenia, And Elevated Transaminases Can Occur Despite Normal 6-Mmp Levels.

***Keywords**: Determining Metabolites; Hplc; Reactive Metabolites; Thiopurines;*

Introduction

Despite Many Advances In The Understanding Of Cancer Biology, Therapeutic Treatment Of Tumors Is Essentially Based On Empirical Approaches. Chemotherapy Carries A High Risk Of Unfavorable Toxicities And Adverse Treatment Outcomes, Due To The Narrow Therapeutic Index Of Antineoplastic Agents. Though, Basic Science Is Continuously Contributing On Improving Treatments' Safety, Efficacy And Quality Through Determination Of Drug Metabolites And Discovering Novel Pharmacogenomic Biomarkers. [1]

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Among Clinically Important Antineoplastic Drugs Are Also Thiopurines. Thiopurines Are Cytostatic, Purine Antimetabolite And Anti-Inflammatory Drug Group Consisting Of Azathioprine (Aza), 6-Mercaptopurine (6-Mp) And 6-Thioguanine (6-Tg). Thiopurines Are Used In The Treatment Of Acute Lymphoblastic Leukemia (All), The Most Prevalent Childhood Cancer, As Well As For Other Autoimmune Diseases And Prevention Of Transplant Rejection. For The Past 40 Years, The Administration Of Daily 6-Mercaptopurine (6-Mp) For 1.5–3 Years, Has Been The Standard 'Backbone' Of All Maintenance Phase Of Treatment. [2,3]

Thiopurines Mechanism Of Action

The Main Mode Of Action Of Thiopurines Is Through Cytotoxicity, Which Is Achieved By Incorporation Of Its Metabolite Thioguanine Nucleotides (Tgn) Into Dna And Rna. The Tgns Can Induce The Apoptotic Cell Death Either By Inhibition Of Intracellular Signaling Pathways Or By Inhibiting Dna Methylation. Another Mode Of Action Of Thiopurines Which Is Demonstrated Only In Vitro On Cell Cultures, Is The Inhibition Of De Novo Purine Synthesis, Through Methylthioinosine Monophosphate (Metimp). [4]

In Contrast, There Are Competing Metabolic Pathways That Can Inactivate 6-Mercaptopurine (6-Mp) To Thiouric Acid And Methyl-Mercaptopurine (Memp), Which Are Catalyzed By Xanthine Oxidase (Xo) And Thiopurine S-Methyltransferase (Tpmt), Respectively. [1]

The Effect Of Metabolism On The Therapeutic Efficacy Of Thiopurines

The Metabolism Of Thiopurines Is Crucial On Treatment Quality, Efficacy And Safety, Namely On Disease Remission, Disease Relapse Rates And As Well On Encountered Adverse Treatment Outcomes.

Thiopurine S-Methyltransferase (Tpmt) Is The Most Important Enzyme On Thiopurine Metabolism.

Tpmt Catalyzes The Methylation, And Such Deactivation Of The 6-Mercaptopurine To Its Inactivated Methyl Metabolite As Methyl-Mercaptopurine (Memp). Thus, Tpmt Through Thiopurine Deactivation Lowers Thiopurines Treatment Efficacy, Lowers Their Cytotoxic Effect And Adverse Treatment Outcomes.

Patients With Low Tpmt Levels Who Are Treated With Standard Doses Of 6-Mercaptopurine, Will More Slowly Deactivate 6-Mercaptopurine And Thus Have Higher Levels Of Thiopurine Metabolite As Thioguanine Nucleotide (Tgn), Which Accumulates In Cancer Cells And Achieves Potentiated Cytotoxic Effect. Thus, Patients With Lowered Tpmt Activity, Respond Better To Thiopurine Therapy By Entering The Remission Phase Faster. Eventhough, These Patients Because Of The Higher Levels Of Tgn Metabolites And Their Cytotoxic Effect, Are Also At Higher Risk Of Developing Adverse Thiopurine Treatment Outcomes Such As Myelotoxicity, Secondary Infections, Stomatitis, Gastrointestinal Disturbances, Hepatitis, Pancreatitis And Secondary Tumors.

Conversely, Patients With High Or Ultrahigh Activity Of Tpmt Enzyme, Who Can Inactivate 6-Mercaptopurine (6Mp) Faster To Its Inactivated Metabolite Methyl-Mercaptopurine (Memp), Can Commonly Lead To Thiopurine Therapy Tolerability, Increased Risk Of Disease Relapse And Increased Risk Of Hepatotoxicity Related To Methylated Metabolites.[5]

Therefore, We Can Clearly Understand The Major Role That The Thiopurine Metabolism Has, Either Through Its Activated Or Deactivated Metabolites, On The Efficacy And Safety Of Thiopurine Treatment Outcomes.

Tpmt Genetic Variations And Their Distribution

The Distribution Of Tpmt Activity In A Healthy Caucasian Population Is Determined To Be Trimodal, With Approximately 90% Of The Population Having Normal Or High Activity, 10% Intermediate And 0.3% Low Or Undetectable Tpmt Activity. Lower Tpmt Activity Is The Consequence Of Mutations In The Tpmt Gene. Although 26 Variant Alleles Have Been Identified, The Most Prevalent And Clinically Significant Are Tpmt*3A (460G>A And 719A>G) And Tpmt*3C (719A>G). Four Tpmt Variants With Decreased Activity (Tpmt*3A, *3B, *3C And *2) Were Detected In More Than 80% Of Caucasians With Low/ Intermediate Tpmt Activity, While The Frequency Of Other Variants Is Extremely Low. [6,7,8,9,10]

These Mutations Appear To Destabilize The Native Structure Of The Tpmt Enzyme, Resulting

In The Formation Of Misfolded Isoforms, Which Subsequently Undergo Rapid Intracellular Tpmt Degradation. Numerous Studies Have Linked The Onset Of Severe Toxicity During Thiopurine Treatment To The Low Tpmt Tpmt Enzyme Activity That Catalyzes Methylation, Thus Deactivation Of 6-Mp.[11]

Determination Of Mutations In The Tpmt Gene Before Starting 6-Mercaptopurine Therapy Constitutes A Fast, Easy And Cost-Efficient Strategy To Individualize Thiopurine Dosing. Therefore, In Order To Avoid Life-Threatening Side Effects In Patients Homozygous For Two Deficient Tpmt Alleles, A 10- 15 Fold Reduction Of The Standard Dose Is Recommended. However, Dose Adjustments For Heterozygous Individuals Are Not As Clearly Defined.[12]

Despite The Relatively High Safety And Efficacy Of 6- Mercaptopurine (6-Mp), Failure Due To Relapse Of Disease And Treatment Related Toxicities Still Occurs In About 20% Of The Treated Patients.

87% Of Relapses Are Isolated In Bone Marrow, 20% Extramedullary To Cns, 10% Extramedullary To Testes And 14% In Combined Locations. In 10–28% Of Patients Therapy Has To Be Discontinued Due To Adverse Events In Patients, While The Life-Threatening Myelosupression Frequency Is Only 1.4 To 5%.

Of Importance To Know, Significant Adverse Thiopurine Treatment Outcomes Are Considered When There Is Dose Reduction Up To 10% And It Lasts For More Than 3 Months, When There Is Discontinuation Of Therapy For More Than 1 Week Or Patient Ends Up Hospitalized.[13]

Novel Pharmacogenomic Biomarkers Explaining Thiopurines' Genotype To Phenotype Discrepancy

Recently, It Has Been Shown That The Pretreatment Determination Of Tpmt Activity Is Not Useful With Respect To Predicting The Efficiency Or Safety Of Thiopurine Therapy In All Patients. Therefore, The Determination Of Mutations In The Tpmt Gene Before Starting 6-Mp Therapy Represents A More Cost-Efficient, Fast And Easy Strategy For Individualizing Thiopurine Dosing And Thus Lowering The Incidence Of Side Effects And Increasing The Efficacy Of The Treatment. However, Concordance Between Genotype E And Phenotype Is Not Perfect, Especially In Tpmt Heterozygous Individuals, Where It Can Be As Low As 50%. For A Certain Number Of Patients, Therefore, The Prediction Of Tpmt Enzyme Activity And Response To Therapy Are Not Possible Purely On The Basis Of Genotype. As A Result, It Is Of Great Importance To Consider Additional Influence Of Biomarkers As Mthfr, Tyms, Itpa, Pacsin2, Nudt15, Which Together With Tpmt Genotype Might Better Predict Responses To Thiopurine Therapy.[14,15]

The First Candidate Is Sam (S-Adenosylmethionine), The Most Important Pharmacometabolomic Marker In Thiopurine Therapy. Sam Is Synthetized From The Essential Amino Acid Methionine And Atp. Sam Stabilizes The 3D Structure Of Tpmt And Increases Its Activity, Thus Sam Reverses The 6Mp Cytotoxicity By Decreasing Thioguanine Nucleotides. [16]

Sam Levels Depend Largely In The Availability Of Folates, Especially On 5,10-Methylenetetrahydrofolate, Which Is Involved Directly In Sam Synthesis. Mthfr (5,10 Methylenetetrahydrofolate Reductase) Catalyzes The Conversion Of 5,10 Methylene Tetrahydrofolate To 5- Methyl- Tetrahydrofolate, The Major Circulating Form Of Folate And The Methyl Donor For Synthesis Of Methionine From Homocysteine. Therefore, Atp, Folates And Mthfr Activity All Have Potential Positive Effects On Sam Levels And Tpmt Activity.[17]

Contrary, Tyms (Thymidylate Synthetase) A Key Enzyme Involved On Dna Replication Catalyzes The Conversion Of Deoxyuridine Monophosphate (Dump) To Thymidine Monophosphate (Dtmp) By Utilizing 5-Methf As A Methyl Donor, Which Leads To Dihydrofolate Recycling. Thus, Tyms Constitutes A Competing Pathway For Mthfr And Correlates Negatively With Sam Levels And Tpmt Activity.[17]

In Addition, Recent Studies On Thiopurines Revealed That The Pacsin2 (A Member Of "Protein Kinase C Family") Plays A Crucial Role On Tpmt Activity As Well. Patients Who Have Pacsin2 Cc Genotype Have Higher Tpmt Activity In Comparison To Patients With Tt Genotype. Furthermore, Pacsin2 Is Related To Myelotoxicity And Gastrointestinal Toxicity Of Patients Treated With 6Mp.[18]

Another Important Biomarker Influencing Tpmt Activity Is Itpa (Inosine Triphosphatase Pyrophosphatase), Since Patients Having At Least One Nonfunctional Allele Of Itpa Are Associated With Longer Event Free Survival (Efs) And Lowered Risk Of Early Bone Marrow Relapse. It Is Possible That The Action Of 6Mp During The Maintenance Phase Is Partially Reversed By The Fully Functional Itpa Activity And This May Then Lead To Survival Of Leukemic Blasts, Resulting In The Relapse Of Disease.[19]

To Conclude, Efficacy And Safety Of Thiopurine Therapy Relies On The Concentration Of The Patient's Cytotoxic Tgn Metabolites, Which In Turn Depends On The Deactivation Of Thiopurines By Tpmt. The Activity Of Tpmt Depends Largely On The Presence Of Genetic Polymorphisms In Tpmt Gene And Other Additional Biomarkers As Mthfr, Tyms, Itpa, Pacsin2, And Nudt15 That Must Acquire Special Attention.

Methods For Determination Of Reactive Metabolites Of Thiopurines

Determining Thiopurine Metabolites Is Crucial On Improving Patients' Safety, Quality And Efficacy Of Treatment. Thiopurine Metabolites Are Used To Measure Patients' Adherence To Therapy, Optimize Thiopurine Dose By Either Finding Safer/Higher Doses If Remission Is Not Achieved, Or By Determining Dose Reductions Without Losing Effectiveness When Adverse Treatment Outcomes Occur. Furthermore, Thiopurine Metabolites Should Be Determined When We Change The Therapy, Notice Elevated Transaminases Influenced From Accumulation Of Inactivated Methyl Entities As 6-Mmp, Or When We Add Adjunctive Allopurinol To Help On Raising The 6-Tg Metabolites And Suppress Formation Of 6-Mmp.[20]

Thiopurine Metabolites Are Measured In Most Laboratories By Determining Their Concentration In Hemolysate. High-Performance Liquid Chromatography (Hplc) Is Used For The Measurements. Thioguanine Nucleotides (Tgn) And The Methylated Thioinosine Derivatives Are Not Measured Directly. Rather, Compounds Actually Measured Are 6-Thioguanine (Tg) For Tgn, And 4-Amino-5-(Methylthio)Carbonyl Imidazole (Amtci) For Methylated Thioinosine Derivatives. 6-Tg Levels Actually Combine 6-Tg, 6-Thioguanosine (Tgr) And Tgn, While Mtci Levels Include 6-Mmp, Mmpr, And 6-Methylthioinosine Nucleotides (Metin). For Both 6-Tg And Amtci, The Measured Levels Include Both Nucleotides And Deoxynucleotides. These Deoxynucleotides Are Present At Low Concentrations, But There Is The Possibility That These Minor Deoxynucleotides May Indeed Play A Larger Role In The Efficacy And Adverse Thiopurine Treatment Outcomes. [20]

The Results Are Reported In Pmol/Rbc. The Reported Reference Range For 6-Tgn Is 235 To 450 Pmol/Rbc, And A Value Of 235 To 450 Is Identified As A Higher Likelihood Of Response To Therapy. A Value <235 Is Reported As Lower Likelihood Of Response, And A Value Above 450 Is Associated With A Higher Risk For Leukopenia. The Reported Reference Range For 6-Mmpn Is <5700, And A Level >5700 Is Thought To Be Associated With A Higher Chance Of Hepatotoxicity, As Presented On The Table 1.[20]

Table 1. The Reference Range For Thiopurine Metabolites [21]

6-TGN	
Suboptimal dosing	<235 pmol 6-TGN/8×108 red blood cells
Optimal dosing	235−450 pmol 6-TGN/8×108 red blood cells
Increasing risk for myelotoxicity and leucopenia	>450 pmol 6-TGN/8×108 red blood cells
6-MMPN	
Hepatotoxicity risk	>5700 pmol 6-MMPN/8×108 red blood cells

A Quick Review On A Validated Hplc Method Used For Monitoring Of Thiopurine Metabolites In Whole Blood

Whole Blood Has To Be Withdrawn From Healthy Volunteers, Who Are Treated With Aza Or 6-Mp Without Any Modification Of The Therapeutic Protocol For At Least Three Months. Patients Should Be Divided Into Three Groups On The Basis Of The Clinical Response To Thiopurine Treatment: Complete Responders (Persistent Remission); Partial Responders (Remission With Occasional Relapses); Non-Responders (Persistent Active Disease). Therapeutic Concentrations Of 6-Tgn And 6-Mmp Are Considered Between 235 And 450 Pmol/Sxlo" Rbc, And Below 5700 Pmol/Sxlo" Rbc Respectively. Possible Drug Toxicity (Hematologic, Hepatic) Can Be Evaluated, Such Hematologic Toxicity Can Be Evaluated With At Least One Of These Three Criteria: A Lymphocyte Count < 1000/Mm3 , A Platelet Count < 100.0001 Mm' And White Blood Cell Count < 40001 Mm', While Hepatic Toxicity Is Considered With Liver Transaminases > 40 U/L.[22]

Sample Preparation

Non-Fasting Peripheral Blood Should Be Collected In Sodium Heparin-Containing Tubes. Initially An Aliquot Of Wb Is Taken And Stored At -20°C Until Analyzed. Afterwards, The Remaining Wb Is Washed To Obtain Purified Rbe. In Particular, Wb Is Centrifuged At 1000Xg For 5 Min At Room Temperature. Plasma And Buffy Coat Are Discarded And Rbc Is Washed Twice With Isotonic Salt Solution (0.9% W/V Nacl). Both Purified Rbc And Wb Are Counted With A Cell Counting Device To Normalize Metabolite Concentrations To Pmol/Sx Lo' Rbc, Aliquoted (500 Ul.) In An Eppendorf Tube And Stored At -20°C Until Analyzed. 25 Ut, Of 120L/Mol/L Ls. And 50 Ul, Of 0.5Mol/L Dtt Are Added To An Aliquot Of Rbc Or Wb. Proteins Are Precipitated With 100 Ul. Of 70% Hci04 , Vortexed For 20 Sec And Held In An Ice Bath. The Tube Is Then Centrifuged For 15 Min At 13,Oooxg At 4°C. The Supernatant Is Transferred Into A New Eppendorf Tube And Heated For 45 Min At 100°E. During This Step 6-Tgn Is Hydrolysed Into Their Corresponding Bases (6- Tg) And In Addition, 6-Mmp Is Converted To 4-Amino5-(Methylthio)Carbonyl Imidazolo (Amtci) . After Cooling, A 100 Ul, Aliquot Is Injected Into The Column For Analysis.[22]

Chromatographic Conditions

Reversed-Phase Chromatography Should Be Performed By Using An Agilent 1200 Series Hplc System Consisting Of A Pump, A Degasser, An Autosampler, A Column Oven And A Uv Detector. Analysis Is Performed On A Synergi 4 11M Polarrp 80A With A Guard Cartridge. Analytes Are Separated By Isocratic Elution At Room Temperature With 1% Acetonitrile In 0.02Mol/L Phosphate Buffer (Ph 3.5). The Total Run Time Is 20 Min, The Flow Rate Was 1Ml/1Min And The Absorbances Are Measured At 341 Nm (6-Tg), 280 Nm (5-Bu) And 304 Nm (6-Mmp Derivative).[22]

Nonetheless, Several Studies Highlighted Significant Problems With The Present Methodology Of Thiopurine Metabolite Determinations And The Reproducibility Of The Assays. Great Variations Occur Depending On How Specimens Are Shipped And Whether They Are Refrigerated. For Example, Median 6-Tgn Concentrations On Day 7 Decrease Significantly To 53% Of Baseline When Kept At Ambient Temperature, Compared With A Smaller Decrease To 90% Of Baseline When Refrigerated.

A Similar Pattern Was Observed For 6-Mmp Measurements. Different Acids Used To Hydrolyze The Nucleotides Before Hplc Will Cause Variation In The Concentrations Of The Metabolites, And Many Are Of The Opinion That Specific Metabolites And Nucleotides Should Be Determined Individually.

There Is Also The Consideration That The Concentration Of The Drug Within The Erythrocyte May Not Fully Correlate With The More Important Concentrations Within Mononuclear Cells. It Is Not Certain That The Measure Concentration Of The Metabolites In Erythrocytes Correlate With The Concentration In Mononuclear Cells.[20]

In Terms Of 6-Tgn Metabolite Level Reproducibility, Studies Revealed That 6-Tgn Levels Were Reproducible With <10% Variability For Patients On Stable Dosing Over A Period Of 2 To 24 Months If Aliquots Were Immediately Frozen And Handled Specifically.[20]

Conclusion

There Are Major Potential Disadvantages Of Testing For Thiopurine Metabolites, Such As The Cost-Versus-Benefit Of The Test, And Mistakes Made Because Of Misinterpretation Or Over-Interpretation Of The Results.

Yet Leaving Expenses Aside, Probably The Most Detrimental Outcome In The Use Of Thiopurine Metabolite Levels Is The Misconception By Both Patients And Healthcare Providers That These Laboratories Provide Assurance That Optimal Dosing Is Being Used To Prevent Complications Of Therapy. Despite The Assumption That Measuring Levels Will Prevent Adverse Effects, Clearly Most Of The Adverse Effects From Thiopurines Are Not Directly Related To 6-Tgn Or 6-Mmp Levels.

Clinical Experience Has Demonstrated That Infections Associated With Thiopurine Use Do Not Regularly Correlate With Leukopenia, And Elevated Transaminases Can Occur Despite Normal 6-Mmp Levels. Clearly, The Target Ranges Of Metabolite Levels Are Only Guidelines, Which Likely Apply To Many Patients, But Not All Patients.[20]

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