Altered systemic bioavailability and organ distribution of azathioprine in methotrexate-induced intestinal mucositis in rats

Sadaf A. Karbelkar, Anuradha S. Majumdar

ABSTRACT

Objective: Intestinal mucositis is a significant problem haunting clinicians for decades. One of the major reasons for its occurrence is high-dose chemotherapy. The study is aimed at investigating effect of intestinal mucositis on pharmacokinetics, organ distribution, and bioavailability of azathioprine (AZA) (6-mercaptopurine).

Materials and Methods: Intestinal mucositis was induced with methotrexate (MTX) (2.5 mg/kg). The oral absorption of AZA and 6-mercaptopurine (metabolite) levels were determined in control and MTX-treated rats: ex vivo (noneverted sac technique) and in vivo (pharmacokinetics and organ-distribution) using high-performance liquid chromatography. Immunohistochemistry was conducted to evaluate peptide transporter expression on luminal membrane of small intestine.

Results: Intestinal permeation of AZA into systemic circulation of rats was lower after MTX administration, widely found in intestinal segments of mucositis-induced rats leading to decline in systemic bioavailability of AZA. Immunohistochemistry findings indicated diminution of peptide transporter expression representing hampered absorption of drugs absorbed via this transporter.

Conclusion: Study outcome has thrown light on altered fate of AZA when administered to individuals with mucositis which suggests modified drug therapy. These findings can further be investigated in different drug classes which might be administered concomitantly in mucositis and study outcome can be further confirmed in mucositis patients in clinical practice also.

KEY WORDS: Azathioprine, bioavailability, intestinal mucositis, organ-distribution, pharmacokinetics

Introduction

Mucositis is an alteration wherein there is painful inflammation and ulceration of mucous membranes lining digestive tract. Mucositis can take place anywhere along gastrointestinal (GI) tract, from mouth termed as oral mucositis to stomach (gastritis) and eventually to the intestines (mostly small intestine) referred to as intestinal mucositis.\(^{[1,2]}\) Intestinal mucositis can be genetically predisposed in certain individuals,\(^{[3]}\) but by and large is an adverse effect of cancer chemotherapy.\(^{[4]}\) Chemotherapeutic agents, apart from acting on cancerous cells, have serious detrimental effects on highly proliferating cells of GI mucous membranes.\(^{[5]}\)

Considering anatomy and physiology of GI tract, mucosa is the innermost layer. Mucositis in GI tract commonly affects small intestine,\(^{[6]}\) which consists of villi majorly involved in absorption of nutrients and drugs. Mucositis affects absorption of drugs, and hence, efficacy of its therapeutic action. Absorption of drugs is influenced by various factors such as drug properties, dose, route, and drug interactions, which may get affected in mucositis.\(^{[7]}\)

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absorption various nutrients. Intestinal mucositis ultimately leads to villous atrophy, i.e., complete destruction of villi caused by immune-mediated reactions.[6] The complete villi destruction leads to decreased plasma citrulline levels. Citrulline is an amino acid produced by villi and also a reliable marker of small bowel damage.[7,8] Intestinal mucositis hampers nutrients absorption required for normal body functioning leading to absorptive dysfunction resulting in malabsorption syndrome.

Along with nutrients, villi are also involved in absorption of various drugs following oral administration, of which majority are normally absorbed via passive diffusion. At times, active transport via specific transporters is a major route of drug absorption in small intestine. Various transporters such as peptide (PepT-1), ion transporters (organic anion transporters), amino acid transporters are involved in absorption process.[10] GI alterations such as mucositis and villous atrophy may alter pharmacokinetic parameters like absorption, bioavailability, and organ distribution of drugs (e.g., peptide drug such as azathioprine [AZA], beta lactam antibiotics, atorvastatin) getting absorbed via these transporters.

Patients suffering from altered GI conditions or undergoing chemotherapy might be on concomitant therapy for treatment of co-morbid conditions such as immunosuppression in organ transplantation, hypertension, and diabetes to name a few. Absorption of these drugs might get altered in mucositis patients where primary site of absorption will be proximal small intestine. Thus, it becomes essential to assess small intestinal absorptive function in drug-induced mucositis patients, with regard to absorption in concomitant oral drug therapy.

Methotrexate (MTX) is a frequently used chemotherapeutic drug found to be effective in various cancer types. Serious side effect of MTX therapy in patients is intestinal mucositis ultimately leading to villous atrophy.[11] Changes in biochemical parameters and plasma citrulline levels because of MTX-induced intestinal mucositis have been reported.[12] The study objective was to explore the effect of MTX-induced intestinal mucositis on systemic bioavailability of AZA, by using ex vivo study to assess changes in permeation and absorption of AZA through proximal small intestinal mucosa and in vivo study to estimate levels of 6-mercaptopurine (6-MP) (active metabolite of AZA) in plasma, intestinal segments, intestinal luminal contents, and liver. The research also focused on effect of MTX administration on PepT-1 activity in luminal membrane of proximal small intestine. AZA is a prodrug used in immunotherapy mostly during organ transplantation and is metabolized in vivo to 6-MP.[13]

The AZA is mainly absorbed via passive diffusion. Herein, we postulated that pathological changes in intestinal mucosa would lead to changes in permeability pattern, pharmacokinetics, bioavailability, and organ distribution of AZA (and 6-MP) ultimately affecting the AZA systemic bioavailability. Hence, the 6-MP levels estimation in vivo was correlated with the extent of absorption, bioavailability, and organ distribution of AZA.

Materials and Methods

**Chemicals**

Mac Chem Pvt. Ltd (Mumbai, India), supplied gift samples of pure MTX and AZA. All other chemicals including AZA tablets (Azoran®) and 6-mercaptopurine monohydrate were obtained commercially.

**Animals**

A total of 30 adult male Sprague-Dawley rats of 180–200 g (Haffkine Bio-pharmaceutical Corporation Ltd., Parel, Mumbai, Maharashtra, India) were used in study. Housed in polypropylene cages with wire-mesh top and husk bedding, rats had access to rat chow and fresh drinking water ad libitum throughout study. They were subjected to 12:12 light and dark cycles at temperature 25 ± 2°C. All experiments were performed in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for the care of laboratory animals (Protocol no. CPCSEA-BGP/2013-01-11).

**Animal Experimentation**

For the purpose of experimentation, all the animals were housed separately as per the respective study groups (MTX-treated and control) in cages.

MTX-treated and control rats were administered MTX 2.5 mg/kg s.c. (dissolved in phosphate-buffered saline [PBS] sterilized by autoclaving) and PBS, respectively, for 3 days.[12]

On day 1, baseline weight was measured and plasma collected for measuring baseline citrulline levels in MTX-treated rats and preserved at −20°C. Baseline citrulline level was compared to post-MTX administration plasma citrulline level on day 4. Food intake and body weights of rats were recorded across 3 study days.

*Ex vivo* evaluations (day 4): After overnight fasting, duodenum and jejunum were dissected after sacrificing rats and noneverted sac technique was conducted to evaluate AZA permeation. A total of 6 rats (n = 3 per group of MTX-treated and control) were used for the *ex vivo* study. Rest of tissue was preserved for histopathological analysis, myeloperoxidase (MPO) activity determination, reduced glutathione (GSH) assay, and immunohistochemistry of PepT-1.

*In vivo* evaluations (day 4): Absorption of AZA was determined by plasma pharmacokinetics and organ distribution in 12 rats in each of these two evaluations (n = 6 per group of MTX-treated and control).

**Food intake and body weight evaluation**

Any change in food consumption and body weights of rats, which were monitored daily for 3 days; was taken as indicators of intestinal mucositis induction.

**Histopathological analysis**

Intestinal tissue specimens (duodenum and jejunum) were cleaned and fixed in 10% buffered formalin and then embedded in paraffin. Processed sections were stained with hematoxylin/eosin and observed under microscope (×100).

**Myeloperoxidase activity determination**

To measure the presence of cellular infiltration, tissue MPO activity was determined by standard enzymatic procedure described previously with slight modifications.[14,15] Briefly, intestinal tissue was homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4). Homogenate was centrifuged and supernatant used for GSH assay. Pellet was used for MPO activity determination spectrophotometrically at 460 nm. Protein concentration of supernatant was determined using Biuret assay.
Reduced glutathione assay

The GSH content was assessed by standardized spectrophotometric assay previously described. The GSH content was expressed as μg/mg tissue protein.

Biomarker (citrulline) analysis

Plasma citrulline level was determined using high-performance liquid chromatography (HPLC) by a method previously described with slight modifications. Briefly, mobile phase (flow rate was 1 ml/min) consisted of 10 mM Phosphate buffer (pH 5.9), acetonitrile and methanol (70:15:15, respectively). Twenty milligrams 5-sulfosalicylic acid was added to 1 ml plasma for protein precipitation. Ten microliters of supernatant was vortexed with 100 μl derivatization reagent (10 mg orthohtaldehyde in 0.5 ml methanol, 2 ml 40 M borate buffer [pH 10.0], and 30 μl 2-mercaptoethanol) and then injected into chromatographic system. Areas of peaks detected by fluorescent detector (excitation wavelength, 338 nm; emission wavelength, 425 nm) were used for quantification. The assay was linear over concentration range studied. Mean correlation coefficient was >0.98.

Ex vivo Study

Noneverted sac technique

Proximal intestinal segments around 7 cm were kept moist in ice-cold, oxygenated buffer pH 7.4. Intestinal sacs filled with 0.5 ml of AZA solution (0.1 mg/ml, prepared in 0.1 N NaOH) were placed in incubation medium (37°C) Krebs-Ringer phosphate buffer saline pH 7.4 (NaCl 0.67%, KCl 0.034%, MgSO4 0.059%, CaCl2 0.011%, Na2HPO4 0.234% w/v, and glucose 0.18% w/v in distilled water). Buffer aliquots were removed at specific time intervals (5–360 min) and analyzed using HPLC.

In vivo study

Plasma pharmacokinetics

On day 4, after overnight fasting, rats were administered AZA (50 mg/kg, p.o.; Azoran® tablets as powder suspended in saline). Then 0.5 ml of blood samples were collected under anesthesia from retro orbital plexus at time 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, and 10 h. Pharmacokinetic parameters (Cmax, Tmax, and area under the curve [AUC]) were determined.

Organ distribution

On day 4, after AZA (50 mg/kg, p.o.) administration, rats were sacrificed at 2 and 6 h. Intestinal (duodenum, jejunum, and ileum) segments and liver were dissected, weighed, and homogenized in PBS pH 7.4 and homogenates were stored at −20°C until further HPLC analysis.

Immunohistochemistry of Peptide Transporter 1

The PepT-1 immunohistochemistry was performed to determine changes in its expression on apical membrane of enterocytes during GI mucositis. Intestinal sections were transferred to three changes of xylene for 30 min, rehydrated with decreasing grades of absolute alcohol, 95%, 70%, 50% and then washed with water. They were autoclaved in citrate buffer pH 6 for antigen retrieval. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. Sections were incubated with PepT-1 antibody for 30 min at room temperature, washed in tris buffer solution pH 7.4, incubated later with horseradish peroxidase (Poly HRP) for 30 min, and ultimately washed in Tris buffer solution pH 7.4. Finally, sections were incubated with 3,3-diaminobenzidine tetrahydrochloride and checked for color change due to antigen-antibody reaction. Sections were washed in Tris buffer solution pH 7.4 and mounted on slide.

Drug Analysis

The HPLC system (JascoChrom NAV; Distributor from Mumbai, India, manufacturing at Japan) with quaternary pump, auto-sampler and photodiode array detector was used for the bioanalysis of AZA and 6-MP in the buffer aliquots, plasma and tissue homogenates. The Hi-Qsil C18 column was used for analysis with column oven set at 25°C.

High-performance Liquid Chromatography Analysis of Azathioprine in Buffer Aliquots

Mobile phase consisted of water (pH 4 using glacial acetic acid) and acetonitrile in ratio 80:20 (flow rate: 1 ml/min). Buffer aliquots were centrifuged and supernatant injected into column. Detector was set at 280 nm. Assay was linear over concentration range 0.1–2.5 μg/ml and mean correlation coefficient was 0.999. Permeability coefficient (Papp) of AZA via small intestinal mucosa was determined using the following formula:

\[ P_{\text{app}} = \frac{dq/dt}{A/C_o} \]

where dq/dt = slope; A = Area; and C_o = initial concentration of the drug solution.

High-performance Liquid Chromatography Analysis of 6-mercaptopurine – Plasma, Intestinal, and Liver Homogenates and Intestinal Luminal Contents

Mobile phase consisted of 0.1% triethanolamine in water (pH 3.5 using orthophosphoric acid) and organic phase (methanol:acetonitrile = 50:50) in the ratio 93:7; flow rate of 1 ml/min. Briefly, 95 μl of plasma, 5 μl of 6-thioguanine (5 ppm; internal standard [IS]; 6-TG), 50 μl DTT, 50 μl water, and 25 μl perchloric acid were mixed. Vortexing it for 2 min, mixture was kept at room temperature for 10 min. Precipitated protein was centrifuged (13,000 rpm for 15 min). Fifty microliters of supernatant was taken in another eppendorf and kept in water bath at 100°C for 45 min. After cooling this solution to room temperature, 20 μl was injected into column. Wavelengths selected were λmax = 323 nm (6-MP) and λmax = 342 nm (6-TG). The assay was linear over concentration range 0.5–5 μg/ml and mean correlation coefficient was 0.998.

In case of tissue homogenates and luminal contents, 100 μl of sample was taken as no suitable IS was found for the assay. The sample was further treated in the same manner as mentioned earlier. Assay was linear over concentration range studied and the mean correlation coefficient was 0.99.

Plasma concentration was expressed as μg/ml. The pharmacokinetic parameters of 6-MP were assessed by standard noncompartmental methods. Maximum concentration in plasma (Cmax) and time to reach the maximum concentration in plasma (Tmax) after oral administration of AZA were read directly from plasma concentration–time plot. Area under plasma concentration–time curve (AUC) was calculated by trapezoidal rule method up to last measured concentration in plasma and was extrapolated to infinity by adding the value of last measured concentration in plasma divided by terminal elimination rate constant (K_el).

Statistical Analysis

Results were expressed as mean ± standard error of mean (SEM). Statistics was applied using GraphPad PRISM 5 software.
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(Demo version, Graph pad Software Inc., San Diego, CA, USA).

Statistical analysis was performed by using t-test to identify significant differences amongst groups. For kinetic and organ distribution data, differences between groups were analyzed with two-way analysis of variance, time and treatment as main factors, followed by postanalysis with Bonferroni’s test. $P < 0.05$ was considered statistically significant when compared with vehicle control group.

**Results**

*Food Intake and Body Weight*

Administration of MTX caused significant difference in average daily food intake in rats causing decrease in their body weight in MTX-treated group. The mean body weight of rats in MTX-treated group was reduced by around 7.6% when compared with vehicle control group [Table 1].

*Histopathological Observations*

Histopathological examination of intestinal tissue from control rats did not reveal any lesions in contrast to MTX-treated rats where severe epithelial damage and inflammatory cellular infiltration was observed [Figure 1].

*Myeloperoxidase Activity*

The MPO activity was measured with a view to evaluating levels of tissue neutrophils capable of damaging tissue. The MTX administration resulted in increased intestinal MPO activity in proximal small intestinal tissue [Table 2].

*Glutathione Levels*

The MTX administration to rats resulted in decreased GSH levels [Table 2], indicating oxidative stress due to MTX.

*Plasma Citrulline Levels*

The MTX administration resulted in significant plasma citrulline levels (9.52 ± 0.83 μM) when compared with baseline citrulline levels (19.31 ± 1.13 μM) before commencing MTX administration ($P < 0.05$, values given in mean ± SEM).

*Effect of Methotrexate-Induced Intestinal Mucositis on Azathioprine Permeation ex vivo and Oral Bioavailability of Azathioprine and its Metabolite 6-Mercaptopurine*

Azathioprine permeation ex vivo (noneveted sac technique)

Levels of AZA permeating through duodenal and jejunal mucosa were lower in MTX-treated rats as compared to vehicle control rats, indicated by HPLC analysis of AZA in buffer aliquots [Figure 2].

*Oral bioavailability of azathioprine and its metabolite 6-mercaptopurine*

*Plasma pharmacokinetics*

Plasma concentration of 6-MP declined significantly in MTX-treated group as compared to vehicle control group. The $C_{\text{max}}$, AUC$_{0-h}$, and AUC$_{0-\infty}$ lowered considerably in intestinal mucositis condition [Figure 3].

*Organ distribution*

Concentration of 6-MP was found to be more in small intestinal (duodenal, jejunal, and ileal) tissue in MTX-treated group as compared to vehicle control group at 2 and 6 h. Since more of 6-MP is retained in duodenal tissue in MTX-treated group, less of it gets permeated through small intestine [Figure 4a]. Similar results were found with respective to 6-MP levels in jejunal and ileal homogenates at 2 and 6 h.

Concentration of 6-MP in liver homogenate in MTX-treated group was found to be less as compared to that in vehicle control group [Figure 4b].

Concentration of 6-MP was found to be more in luminal contents of vehicle control group (84.14 ± 6.823 μg/ml) as compared to MTX-treated group (22.36 ± 1.489 μg/ml) at the end of 6 h. Since more of 6-MP is retained in intestinal tissue in intestinal mucositis after MTX administration, less of it remains in small intestinal lumen for permeation through enterocytes into the systemic circulation.

*Immunohistochemistry of Peptide Transporter 1*

Differences in PepT-1 expression on apical membrane of the villi in duodenum and jejunal of vehicle control and MTX-treated rats were observed [Figure 5]. The effect of MTX administration on enterocytes was in agreement with the features observed in histopathological analysis of duodenum and jejunum in vehicle control and MTX-treated groups.

**Discussion**

Intestinal mucositis is a major concern of any chemotherapeutic regimen haunting clinicians for years. In our research work, we have standardized a rodent model simulating small intestinal mucositis experienced by high-dose chemotherapy patients. Major side effect of chemotherapy is deteriorating effect on GI mucosa. The MTX acts on rapidly dividing cells of GI mucosa causing apoptosis by inhibition of DNA synthesis.[22]

Findings of our study are in agreement with mentioned effect of MTX on small intestine. Resulting villous atrophy of proximal small intestine revealed by histopathological analysis of intestinal specimens of MTX-treated rats, showed the presence of necrosis, shortened villi, exocytosis and total villous collapse as compared to vehicle control rats. Marked

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### Table 1:

| Parameter | Control | MTX-treated |
|-----------|---------|-------------|
| Body weight change (%) | 2.93±0.25 | −3.30±0.36* |
| Average food intake per rat per day (g) | 38.53±0.79 | 28.67±0.87* |

*P<0.05. Values are expressed as mean±SEM (n=12). MTX=Methotrexate, SEM=Standard error of mean

### Table 2:

| Group      | Tissue | MPO activity (units/g tissue weight) | GSH levels (μg/mg tissue protein) |
|------------|--------|------------------------------------|----------------------------------|
| Control    | Duodenum | 13.37±0.2 | 2.85±0.37 |
|           | Jejunum | 2.93±0.25 | 1.78±0.2* |
| MTX-treated | Duodenum | 46.03±1.81 | 1.72±0.2* |
|            | Jejunum | 41.22±2.42 | 1.65±0.06* |

*P<0.05. Values are expressed as mean±SEM (n=3). MPO=Myeloperoxidase, MTX=Methotrexate, GSH=Glutathione, SEM=Standard error of mean
Increase in oxidative stress in intestinal tissue of MTX-treated rats was pointed out by lower GSH levels obtained. The striking increase in MPO activity in intestinal tissue indicating neutrophil infiltration was also seen in MTX-treated rats.

Citrulline, a nonprotein amino acid, is a marker of reduced bowel mass. Thus, upon intestinal mucositis progression, the apoptosis of enterocytes causes lowering of plasma citrulline levels. After MTX administration, significant decrease in plasma citrulline levels was observed which is in affirmation with our histopathological findings.

The study rationale was to determine any changes in absorption pattern and organ distribution of prodrug AZA, if it is been administered in drug-induced intestinal mucositis. Drugs like AZA (6-MP) which are purine analogues are majorly absorbed from intestinal lumen via passive diffusion and a specific transport mechanism [PepT1]. Mucosal damage caused by MTX leads to cell death in small intestinal apical membrane causing perforations in tight junctions of enterocytes. Hence, intestinal integrity is lost, resulting in sinking in of AZA (and 6-MP) into enterocytes. Lipophilic nature of AZA (6-MP) also contributes to diffusion in intestinal tissue. Purines are rapidly taken up by cells and cell organelles for incorporation into DNA leading to DNA repair in damaged enterocytes. Therefore, in MTX-treated rats most of the AZA absorbed via passive diffusion, remains into intestinal tissue and less of it gets permeated into systemic circulation. As a consequence, levels of 6-MP found in intestinal tissue of MTX-treated group were higher as compared to vehicle control group. Subsequently, the plasma concentration of 6-MP was found to be lower in MTX-treated group.
The AZA is indicated majorly as an immunosuppressant in organ transplantation. Hence, it has to be available in sufficient levels systemically to show its action. Study findings suggest reduced efficacy of AZA given concomitantly for immunotherapy in individuals on chemotherapy in drug-induced mucositis. Additionally in order to get more insights into effect of intestinal mucositis and villous atrophy on drug bioavailability, any absorptive dysfunction should be assessed in clinical practice.

We have focused on PepT-1 transporter in the present study, since it is present on apical brush-border membrane of enterocytes. This transporter is majorly involved in absorption of various di-and tri-peptides from intestinal lumen. Immunohistochemistry of duodenum and jejunum revealed that there is reduced expression of PepT-1 transporter on apical membrane of villi in MTX-treated rats as compared to vehicle control rats. Diminished PepT-1 expression is mainly seen in duodenum and slightly in jejunum of MTX-treated rats. The diminished PepT-1 expression causes deficiency of various dietary proteins/peptides which are absorbed via PepT-1 from intestinal lumen leading to malabsorption syndrome. In addition, there might be hampered effect on drugs’ absorption via PepT-1 from gut lumen. As a result, there might be reduced efficacy of drugs which are PepT-1 substrates and have to be administered in any co-morbid conditions.

Recently, research has focused on Peptide Transporter-Associated Prodrug Therapy via small intestinal membrane. These studies have explored various amino acid prodrugs of valcyclovir and AZA that have advantageous property of improved oral absorption. These prodrugs are transported majorly via PepT-1 localized in proximal apical brush border membrane of intestinal villi. Thus, if such prodrugs are administered to chemotherapeutic patients, it might lead to hampered absorption of these. This would also lead to lower tissue concentrations, as transport of such prodrugs would primarily be PepT-1 dependent. The lower plasma concentration of the active moiety, consequently leading to inappropriate systemic and tissue levels would cause inefficacious therapeutic regimen eventually.

This preclinical study has unraveled the detrimental effect of MTX-induced intestinal mucositis on systemic bioavailability of AZA. The effect of intestinal mucositis can be studied on different other drugs in order to understand their fate when given in intestinal mucositis conditions.

In conclusion, because of GI and PepT-1 expression profile alteration, modification in AZA systemic bioavailability was detected. Eventually, drug-induced intestinal mucositis developed after MTX administration had plasma pharmacokinetic consequences. The study can also give insights about changes in absorption pattern and bioavailability of similar drugs caused by absorptive dysfunction in case of GI alterations. This study also drives us to request clinicians to substantiate the impact of intestinal mucositis on systemic bioavailability of relevant drugs.

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Conflicts of Interest
There are no conflicts of interest.
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