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Original Article

Toxicity Evaluation of 6-Mercaptopurine Using Accelerated Cytotoxicity Mechanism Screening (ACMS) techniques

Ahmad Salimi[a,b,c], Melina Rahmandoost[a], and Jalal Pourahmad[a]*

[a] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

[b] *Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Science, Ardabil, Iran*

[c] *Students Research Committee, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

Abstract

6- Mercaptopurine (6-MP) is widely used in clinic as an immunosuppressive for treatment of acute lymphocytic leukemia, Crohn's disease, and ulcerative colitis with documented unpredictable hepatotoxicity. The potential molecular cytotoxic mechanisms of 6-MP against isolated rat hepatocytes were searched in this study using "Accelerated Cytotoxicity Mechanism Screening (ACMS)" techniques. The concentration of 6-MP required to cause 50% cytotoxicity in 2 hour at 37◦C was detected to be 400 µM. A significant increase in 6-MP induced cytotoxicity and reactive oxygen species (ROS) formation, % mitochondrial membrane potential (MMP), lysosomal damage were observed. The addition of chloroquine (lysosomotropic agent), L-carnitine (inhibitor of membrane permeability transition (MPT), Diphenyleneiodonium (DPI) as an inhibitor of production of superoxide, and H_2O_2 by mitochondria and Dimethyl sulfoxide (DMSO) as a radical scavenger decreased 6-MP-induced cytotoxicity, ROS formation, collapse of MMP, and lysosomal damage. Results from this study suggest that 6-MP -induced cytotoxicity in isolated rat hepatocytes due to ROS formation, mitochondrial and lysosomal damages that resulted in crosstalk toxicity between mitochondrial and lysosomal damage and finally cell death.

Key words: ACMS**,** Hepatotoxicity, lysosome, Mercaptopurine, Mitochondria, Reactive Oxygen Species.

1. Introduction

Drug-induced liver injury (DILI) is a main concern in clinical studies and also in post-marketing surveillance of drugs [1]. Three thiopurine drugs have been commonly used in the last forty years such as 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and azathioprine (AZA) are widely used for the treatment of

Corresponding Author: Jalal Pourahmad, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.Tel: +98(21)2255-8786
E-Mail: j.pourahmadjaktaji@utoronto.ca
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diseases such as autoimmune conditions, inflammatory bowel diseases (IBD), following transplantation to avoid organ rejection and acute leukemia [2]. In most of the cases, hepatotoxicity is an unforeseeable side effect of 6-MP, whose pathogenic mechanism remains unknown [3]. Hepatotoxicity is a relatively unusual but important complication of 6-MP [4]. It is revealed by raising in aminotransferases and often accompanied by flu-like symptoms including headaches, nausea, and fatigue almost resolved after 6-MP discontinuation or dose reduction [5]. In some patients, however, a syndrome of liver vascular disorders or isolated cholestasis can be seen [6]. Measurement of the metabolite of 6-MP (6-MMPR) has been suggested as a means to recognized patients at risk for hepatotoxicity. Several studies have shown a positive relationship between the elevated metabolite of 6-MP levels and abnormal liver function tests in the pediatric population [7].

6-MP is a chose drug for treatment of inflammatory disorders. Moreover, previous studies suggests that some drugs during periods of inflammation can increase an individual's susceptibility to toxicity [8]. Inflammation caused by endotoxins or infections markedly activates NADPH (nicotinamide adenine dinucleotide phosphate) oxidase that produces superoxide radicals by transferring electrons from NADPH. In the phagosome, superoxide radicals automatically generate form hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) [9]. Finally drug toxicity has been cited as the most common reason for discontinuation of 6-MP therapy [10]. The aim of this study was study of toxic pathways 6-mercaptopurine in rat hepatocytes using the "Accelerated Cytotoxicity Mechanism Screening" techniques.

2. Materials and Methods

2.1. Animals

Male Sprague–Dawley rats weighing 250–300 g were housed in a room at a stable temperature of 25^{o} C on a12/ 12 h light/dark cycle with water and food accessible *ad libitum* . All examinations were conducted according to ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.2. Isolation of Hepatocytes

Hepatocytes were received by collagenase perfusion of the liver. Viability of isolated hepatocytes was measured by trypan blue (0.2 w/v) exclusion test. Hepatocytes were suspended

at a density of 10^6 cells/mL in round-bottomed flasks rotating in a water bath maintained at 37º C in Krebs-Henseleit buffer (pH = 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% O_2, 85% N_2, and 5% CO_2. Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period. At least 80–90% of the control cells were still viable after 2 h [11].

2.3. Viability Assessment

Hepatocyte viability was tested microscopically by plasma membrane disruption, as determined by the trypan blue (0.2% w/v) exclusion test. Hepatocyte viability was assessed every 30 min during the 3 h incubation period, and the hepatocytes were at least 80–90% viable before use. To avoid either non-toxic or severe toxic conditions in this study, EC_{50} 2h concentrations were used for 6-MP. The EC502h of a chemical in hepatocyte cytotoxicity assessment technique is defined as the concentration, which decreased the hepatocyte viability to 50% following the 2 h incubation period [11].

2.4. ROS Assay

Hepatocyte reactive oxygen species (ROS) formation induced by the 6-MP was detected by adding dichlorofluorescin diacetate (DCFD). DCFD interpenetrates to hepatocytes and is hydrolyzed to non- fluorescent dichlorofluorescin. Dichlorofluorescin is then oxidized by ROS to form the highly fluorescent dichlorofluorescein which effluxes the cell. After incubation with 6-MP, 1000 µL samples of cells were withdrawn at 30 and 60 min and centrifuged at 50 rpm for 1 min. The cells were suspended in Krebs–Henseleit buffer and 1.6 µM DCFD was added. Hepatocytes were allowed to incubate at 37°C for 10 min. Excitation and emission wavelengths were 490 and 520 nm, respectively[11].

2.5. MMP Assay

The uptake and retention of the cationic fluorescent dye, rhodamine 123, has been used for the assessment of MMP collapse. This measurement is based on the fact that rhodamine 123 accumulates selectively in the mitochondria by facilitated diffusion. However, when the mitochondrial potential is decreased, the amount of rhodamine 123 that enters the mitochondria is also decreased as there is no facilitated diffusion. Thus, the amount of rhodamine 123 in the supernatant is increased and the amount in the pellet is decreased. Samples (0.5 ml) were taken from the cell suspension incubated at 37 °C at different time points, and centrifuged at 50 × g for 1 min. The cell pellet was then resuspended in 2 mL of fresh incubation medium containing 1.5 µM rhodamine 123 and incubated at 37 °C in a thermostatic bath for 10 mins with gentle shaking. Cells were separated by centrifugation and the amount of rhodamine 123 appearing in

the incubation medium was measured fluorimetrically, using Hitachi F-2500 fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference in fluorescence intensity between control and treated cells [11].

2.6. Lysosomal Membrane Integrity Assay

Hepatocyte lysosomal membrane stability was measured from the redistribution of the fluorescent probe, acridine orange. Aliquots of the hepatocytes suspension (0.5 mL) that were previously incubated with acridine orange (5 μM) were separated from the incubation medium by 1 min centrifugation at 1000 rpm. The hepatocyte pellet was then resuspended in 2 mL of incubation medium. This washing process was repeated for two times to remove the fluorescent dye. Acridine orange redistribution in the hepatocytes suspension was then measured fluorimetrically Hitachi F-2500 fluorescence spectrophotometer set at excitation and emission wavelengths 490 and 520 nm, respectively [11].

2.7. Statistical Analysis

Results are presented as means ± SD. All statistical analyses were performed using Graph Pad Prism (version 5, Graph pad Software Inc.). Assays were performed in triplicate and the mean was used for the statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test. In some experiments, the two-way ANOVA test, followed by the post-hoc Bonferroni test was also performed. Statistical significance was set at $P < 0.05$.

3. Results and Discussion

3. 1. Results

3.1.1. Cytotoxicity

The EC50, 2h concentration found for 6-Mp was equivalent to 200 μM and. As shown in figure 1, 6-MP significantly decreased hepatocyte viability compared to control hepatocytes ($P < 0.05$). 6-MP induced cytotoxicity was significantly ($P < 0.05$) prevented by chloroquine, L-carnitine, DPI, and DMSO.

3.1.2. ROS Formation

Incubation of hepatocytes with 6-MP (200, 400 and 800 μM) caused a significant increase in ROS formation as compared with control hepatocytes ($P < 0.05$) (Table 1). Pretreatment of hepatocytes by different agents such as chloroquine, L-carnitine, DPI and DMSO showed protection against 6-MP induced ROS formation in a time-dependent manner (Table 1).

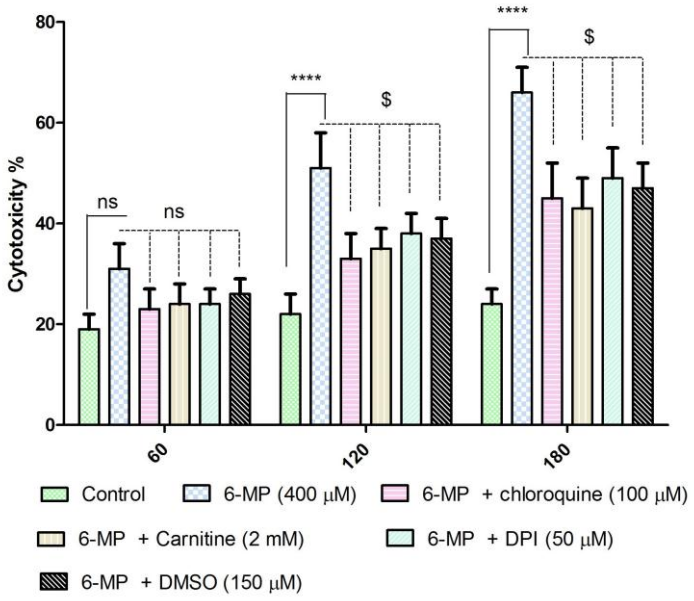

Figure 1. Effect of ROS scavengers, MPT pore sealing agents, lysosomotropic agents, and NADPH P450 reductase inhibitor on 6-MP-induced hepatocyte toxicity. Hepatocytes (10^6 Cells/mL) were incubated in Krebs–Henseleit buffer with pH of 7.4 at 37° C for 1.0 h following the addition of 6-MP. Cytotoxicity was determined as the percentage of cells that uptake trypan blue. Values are expressed as mean ± SD of three separate experiments (n = 3).

***: Significant difference in comparison with control hepatocytes ($p < 0.05$);

$$$: Significant difference in comparison with 6-MP treated hepatocytes ($p < 0.05$)

3.1.3. MMP Collpase

As shown in table 2, 6-MP (200, 400 and 800 μM) also caused a rapid decline of mitochondrial membrane potential as compared with control hepatocytes ($P < 0.05$) which was pre-vented by different agent such as chloroquine, L-carnitine, DPI, and DMSO.

3.1.4. Lysosomal Damage

When hepatocyte lysosomes were loaded with acridine orange (a lysosomotropic agent), a significant redistribution of acridine orange into the cytosolic fraction ensued within 30 and 60 min of incubation with 6-MP. 6-MP induced acridine orange release were again prevented by chloroquine, L-carnitine, DPI, and DMSO indicating sever oxidative damage to lysosomal membrane (Table 3).

3.2. Discussion

Drug hepatoxicity can be nonidiosyncratic (predictable) or idiosyncratic (unpredictable).

New epidemiologic data suggest that approximately 20 new cases of DILI per 100,000 persons occur each year. Idiosyncratic DILI accounts for 11% of the cases of acute liver failure [3]. Previous evidence suggests that drug exposure during periods of inflammation can increase an individual's susceptibility to toxicity [12]. Mercaptopurine, one of drugs that used form inflammatory disorder is believed to have a direct, similar types of injury and dose related hepatotoxic effect can be reproduced in animal studies [13, 14]. The hepatotoxic effects of mercaptopurine, have been linked to higher levels of methyl-mercaptopurine, a metabolite of mercaptopurine that is produced during metabolism. Also the acute cholestatic hepatitis associated with mercaptopurine, is more likely due to idiosyncratic reaction [15].

Previous studies have demonstrated that azathioprine a parent compound of 6-MP decreased the viability of rat hepatocytes [16]. Our study shows the mechanism by which clinically relevant concentrations of 6-MP (200-800 µM) damage rat isolated hepatocytes. In primary studies, 200, 400, and 800 µM concentrations of 6-MP were toxic, and 6-MP profoundly decreased cell viability; further, cytotoxicity was prevented by lysosomotropic agent (Chloroquine), MPT pore sealing agents (Carnitine), NADPH P450 reductase inhibitor (DPI) and hydroxyl radical scavenger (dimethyl sulfoxide) (Figure 1). All of these reagents did not show any effects on hepatocyte toxicity at concentrations used (data not shown).

Our findings regarding oxidative stress parameters revealed that 6-MP could increase

Table 1. Effect of ROS scavengers, MPT pore sealing agents, lysosomotropic agents, and NADPH P450 reductase inhibitor on 6-MP-induced hepatocyte toxicity, ROS formation.

Addition	**ROS**					
	200 µM		400 µM		800 µM	
	30 min	60 min	30 min	60 min	30 min	60 min
Control	152 ± 11.4	154 ± 9.4	430 ± 13.3	454 ± 12	468 ± 9.4	526 ± 10.4
6-MP	152 ± 12.2	160 ± 8.7[a]	583 ± 14.1[a]	592 ± 11.7[a]	669 ± 13.1[a]	716 ± 12.4[a]
+Chloroquine (100 µM)	125 ± 6.4[b]	150 ± 3.5[b]	431 ± 10[b]	461 ± 9.5[b]	576 ± 11.5[b]	706 ± 6.4
+Carnitine (2 mM)	125 ± 6.1[b]	127 ± 2.8[b]	527 ± 9.4[b]	570 ± 11.3[b]	496 ± 10.6[b]	532 ± 8.4[b]
+DPI(50 µM)	145 ± 7.1[b]	143 ± 6.4[b]	448 ± 8,6[b]	538 ± 10.3[b]	551 ± 9.2[b]	574 ± 9.7[b]
+DMSO (150 µM)	145 ± 4.2[b]	143 ± 5.4[b]	441 ± 11.2[b]	458 ± 12[b]	572 ± 11.4[b]	706 ± 9.4

Hepatocytes (10^6 Cells/mL) were incubated in Krebs–Henseleit buffer with pH of 7.4 at 37° C for 1.0 h following the addition of 6-MP. Cytotoxicity was determined as the percentage of cells that uptake trypan blue. DCF formation was expressed as fluorescent intensity units. Values are expressed as mean ± SD of three separate experiments (n = 3).

a : Significant difference in comparison with control hepatocytes ($p < 0.05$);

b : Significant difference in comparison with 6-MP treated hepatocytes ($p < 0.05$).

ROS production. The increased ROS formation may play a significant role in the enhancement of lipid peroxidation and finally cellular and sub cellular mem-brane damage. Pervious investigation revealed that clinically relevant concentrations of 6-MP and azathioprine are toxic to rat hepatocyte cultures by a mechanism that involves oxidative stress, mitochondrial injury, and ATP depletion [17].

Mitochondria are involved in the generation of ROS through one-electron carriers in the respiratory chain; mitochondrial structures are also very susceptible to oxidative stress [18]. Our results proved that 6-MP could cause mitochondrial damage which could be prevented by lysosomotropic agent (Chloroquine), MPT pore sealing agents (Carnitine), NADPH P450 reductase inhibitor (DPI), and hydroxyl radical scavenger (dimethyl sulfoxide) (Table 2). Also mitochondria damages may lead to release of proapoptotic agents from mitochondria to cytosol and finally induce apoptosis through mitochondria pathways [19] related to oxidative stress induced by 6-MP. Previous study showed that azathioprine a parent compound of 6-MP involves depletion of GSH leading to mitochondrial injury with profound depletion of ATP and cell death that was prevented by potent antioxidants, glycine and blocking the mitochondrial permeability transition pore [16].

Table 2. Effect of 6-MP, ROS scavengers, MPT pore sealing agents, lysosomotropic agents, and NADPH P450 reductase inhibitor on 6-MP-induced mitochondrial injury in hepatocytes

Addition	**MMP collapse**					
	200 µM		400 µM		800 µM	
	30 min	60 min	30 min	60 min	30 min	60 min
Control	2329 ± 21.5	2480 ± 27.8	2390 ± 23.6	2593 ± 32.2	2382 ± 27.8	2450 ± 40.6
6-MP	3117 ± 24.2[a]	3493 ± 21.7[a]	3631 ± 24.8[a]	3973 ± 36.6[a]	5080 ± 33.1[a]	5693 ± 32.8[a]
+Chloroquine (100 µM)	2435 ± 18.4[b]	2632 ± 19.1[b]	2737 ± 26.9[b]	2874 ± 24.8[b]	3245 ± 37.5[b]	3833 ± 29.4[b]
+Carnitine (2 mM)	2423 ± 16.1[b]	2641 ± 23.8[b]	2768 ± 29.3[b]	2643 ± 17.8[b]	3304 ± 20.6[b]	3754 ± 28.7[b]
+DPI(50 µM)	2440 ± 19.3[b]	2664 ± 27.3[b]	2775±32.6[b]	2947 ± 30.4[b]	3137 ± 27.5[b]	3932 ± 29.4[b]
+DMSO (150 µM)	2408 ± 23.1[b]	2601 ± 31.2[b]	2530 ± 33.6[b]	2643 ± 37[b]	3002 ± 34.4[b]	3635 ± 41.4[b]

Hepatocytes (10^6 Cells/mL) were incubated in Krebs–Henseleit buffer with pH of 7.4 at 37° C for 1.0 h following the addition of 6-MP. Cytotoxicity was determined as the percentage of cells that uptake trypan blue. MMP collapse was expressed as fluorescent intensity units. Values are expressed as mean ± SD of three separate experiments (n = 3).
a: Significant difference in comparison with control hepatocytes ($p < 0.05$);
b : Significant difference in comparison with 6-MP treated hepatocytes ($p < 0.05$).

Table 3. Effect of 6-MP, ROS scavengers, MPT pore sealing agents, lysosomotropic agents, and NADPH P450 reductase inhibitor on lysosomal integrity, in isolated hepatocytes.

Addition	**Lysosomal integrity**					
	200 µM		400 µM		800 µM	
	30 min	60 min	30 min	60 min	30 min	60 min
Control	1016 ± 15.7	1236 ± 17.9	1579 ± 19.6	1695 ± 12.2	1281 ± 18.8	1378 ± 20.1
6-MP	3043 ± 18.5[a]	3118 ± 20.5[a]	3453 ± 14.3[a]	4159 ± 16.8[a]	4031 ± 13.1[a]	4464 ± 22.9[a]
+Chloroquine (100 µM)	1649 ± 21.5[b]	2106 ± 13.1[b]	2327 ± 18.9[b]	2483 ± 9.8[b]	2021 ± 17.5[b]	2402 ± 19.4[b]
+Carnitine (2 mM)	1916 ± 26.3[b]	2238 ± 21.1[b]	1822 ± 19.3[b]	1945 ± 27.4[b]	2176 ± 18.6[b]	2491 ± 18.5[b]
+DPI(50 µM)	1698 ± 12.9[b]	1836 ± 17.4[b]	1742±12.6[b]	2278 ± 10.4[b]	2160 ± 17.5[b]	2360 ± 19.8[b]
+DMSO (150 µM)	2025 ± 13.6[b]	2250 ± 9.8[b]	2323 ± 23.6[b]	2461 ± 17.8[b]	1942 ± 14.4[b]	2017 ± 11.4[b]

Hepatocytes (10^6 Cells/mL) were incubated in Krebs–Henseleit buffer with pH of 7.4 at 37° C for 1.0 h following the addition of 6-MP. Cytotoxicity was determined as the percentage of cells that uptake trypan blue. Lysosomal integrity was expressed as fluorescent intensity units. Values are expressed as mean ± SD of three separate experiments (n = 3).
a: Significant difference in comparison with control hepatocytes ($p < 0.05$);
b : Significant difference in comparison with 6-MP treated hepatocytes ($p < 0.05$).

When hepatocyte were loaded with acridine orange ensued within 30 and 60 min the loaded hepatocytes were treated with 6-MP, indicating leakiness of the lysosomal membrane (Table 3). On the other hand, the 6-MP-induced acridine orange release was prevented by the lysosomotropic agent (chloroquine), MPT pore sealing agents (carnitine), NADPH P450 reductase inhibitor (DPI), and hydroxyl radical scavenger (dimethyl sulfoxide), suggesting involvement of pH- dependent intralysosomal Fenton's type reactions and ROS formation in 6-MP-induced lysosomal membrane damage in hepatocyte [20].

4. Conclusion

All our results were in favor of the entanglement of the ROS formation hypothesis in 6MP-induced hepatocyte cytotoxicity, since both lysosomal membrane labilization and the decline in MMP were attended following the 6-MP-induced ROS formation and cytotoxicity in hepatocytes. It is supposed that the ROS formation causes damage on lysosomal and mitochondrial membranes through lipid peroxidation, which finally leads to cell death [21].

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