Alterations of Apoptotic and Epigenetic Genes Associated with Gatifloxacin-Induced Oxidative Stress in Rat Liver

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Abstract

In order to investigate the alterations in the expression of genes involved in epigenetics and apoptosis associated with gatifloxacin-induced oxidative stress in rat liver, adult rats were exposed to 10 mg/kg, 20 mg/kg, 40 mg/kg and 80 mg/kg gatifloxacin for five days orally. Biomarkers of oxidative stress were assessed spectrophotometrically while the levels of expression of Bcl2l1, caspases 3, 8 and 9 as well as Dnmt1, Hdac5, Prdm2, Eid3, Suv39h1 and Ehmt2 were assessed using relative reverse transcription polymerase chain reaction. The results showed that the dose-dependent increase in oxidative stress was associated with increase in the expression of proapoptotic genes. Gatifloxacin treatment also resulted in significant ($p < 0.05$) increase in the expression level of DNA and histone methylating genes. These changes observed at the lowest dosage of 10 mg/kg showed that gatifloxacin exposure could result in apoptosis and trigger epigenetic changes in the liver.

Keywords: word; Gatifloxacin, oxidative stress, epigenetics, apoptosis

1. Introduction

Gatifloxacin (1-Cyclopropyl-1,4-dihydro-6-fluoro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid, DB01044) is a member of the fourth-generation fluoroquinolone antibiotic family that is used in treating infection caused by a broad range of microorganisms. It functions by inhibiting the bacterial enzymes DNA gyrase and topoisomerase IV in Gram-positive and Gram-negative organisms, including anaerobes such as, *Mycoplasma*, *Chlamydia*, and *Legionella* and mycobacteria [1]. Fluoroquinolones, including gatifloxacin, have been reported to produce several side effects including hepatotoxicity, joint defects and phototoxicity with complications like liver damage, purpura and dysglycemia [2-4]. In particular, gatifloxacin has been reported to induce fulminant hepatic failure [3]. Olayinka, Ore [5] reported that exposure of rats to

graded doses of gatifloxacin resulted in liver damage characterized by hepatic portal congestion and cellular infiltration by mononuclear cells as well as elevation in the activities of plasma biomarkers of liver damage like alkaline phosphatase, alanine transaminase, aspartate aminotransferase and gamma-glutamyl transferase. These side effects like phototoxicity, cartilage damage and liver damage have been linked to the generation of reactive oxygen species (ROS) leading to oxidative stress [6]. Fluoroquinolones penetrate neutrophils and enhance their antimicrobial activity by generating ROS [1]. Although studies have shown the potential of gatifloxacin to induce oxidative stress, there is dearth of information on whether the induced oxidative stress alters the expression of genes involved in oxidative DNA damage/repair.

Evidences are now emerging that oxidative stress is accompanied with changes in epigenetic signature of the DNA in the liver and that xenobiotics can modulate these changes [7, 8]. Epigenetic modifications are modifications affecting the expression of DNA without affecting the DNA sequence. These modifications include DNA methylation and histone modifications [9]. Although it is becoming well-established that various agents can cause epigenetic changes, there is still a dearth of information on the ability of pharmaceuticals to induce epigenetic changes. A recent study has suggested gatifloxacin as an agent that can alter pluripotency by interfering with histone modification signature [10].

Therefore, to further elucidate the mechanism of gatifloxacin-induced toxicity in the liver, this study investigated the effect of gatifloxacin on oxidative stress and expression of genes associated with apoptosis, DNA methylation and histone modification in rat liver.

1. Material and methods

2.1 Chemicals and reagents

Gatifloxacin was obtained from Sigma-Aldrich, St. Louis, MO. EASYspin Plus® was obtained from Aidlab Biotechnologies Co., Ltd, Beijing, China while RNAhold® and *EasyScript*® one-step RT-PCR kit was obtained from TransBionovo Co., Ltd. Beijing, China. Other chemicals and reagents were obtained from Sigma-Aldrich, St. Louis, MO.

2.2 Animals and experimental procedure

Twenty-five (25) inbred male albino rats weighing 130±30 g were used for this research. The animals were subjected to standard 12-h light and dark cycles and provided water and feed *ad libitum*. The animals were allowed to acclimatize for two (2) weeks before starting the experiments and they were randomly distributed into five (5) groups. Group 1 served as control, while the remaining groups received varying doses of gatifloxacin thus: group 2 (10 mg/kg bw), group 3 (20 mg/kg bw), group 4 (40 mg/kg bw) and group 5 (80 mg/kg bw) orally for 5 days. The rats were sacrificed 24 hours after the last administration under light ether anaesthesia and liver was excised immediately. The liver samples for oxidative stress assays were processed appropriately [11] while portions of the liver were kept in RNAhold® and stored at -80 °C for RNA analysis.

2.3 Biochemical analysis

The level of lipid peroxidation was determined by assessing the concentration of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust [12]. Glutathione-S-transferase's activity was determined according to the method of Habig [13]. Superoxide dismutase's activity was determined according to the

method of Marklund and Marklund [14]. Glutathione concentration was determined according to the method of Ellman [15]. Nitric oxide (NO) concentration was determined by the Griess reaction using a method described by Yucel *et al.,* [16]. The Lowry method was used for the determination of protein concentration as described by Gallagher and Desjardins [17].

The tissue level of H_2S was assayed using the methylene blue formation method as described Shen et al [18]. Briefly, 75 µL of liver homogenate was mixed with 250 µL Zn acetate (1%) and 450 µL distilled water for 10 min at room temperature. TCA (10%; 250 µL) was then added, centrifuged at 14,000 g for 10 min and the clear supernatant was mixed with N,N-dimethyl-p-phenylenediamine sulfate (20 mM/L; 133 µL) and $FeCl_3$ (30 mM/L; 133 µL). The absorbance was read at 670 nm after 20 min.

2.4 Gene expression analysis

The expression level of certain apoptotic, DNA methylating and chromatin modifying genes
(Table 1) were assessed using relative reverse transcriptase polymerase chain reaction (RT-PCR) techniques as described by Chaudhry [19],with appropriate modifications. In brief, RNA was extracted from the liver using Aidlab® EASYspin Plus® kit according to the manufacturer's instructions. The RT-PCR was carried out with 500 ng RNA template using the Transgen® *EasyScript*® one-step RT-PCR reagent according to the manufacturer's instructions. Samples were subjected to an initial incubation at 45^oC for 30 minutes for cDNA synthesis, followed by PCR amplification, using gene specific primers (GSP) (Table 1), 94^oC for 5min followed by 40 cycles of 94^oC for 30s, 5min at the annealing temperature of GSP and 1min at 72^oC. All amplifications were carried out in

C1000 Touch™ Thermal Cycler (BioRad, CA, USA).

The level of transcription of the genes relative to β-actin was quantified using Image J® software [20, 21].

2.5 Statistical analysis

Data was expressed as mean ± SEM of six replicates in each group. Analysis of variance (ANOVA) was carried out to test for the level of homogeneity at $p < 0.05$ among the groups. Duncan's multiple range test was used to separate the heterogeneous groups.

2. Results

3.1 Gatifloxacin induced oxidative stress in rat liver

The levels of GSH, H_2S, TBARS and NO as well as the activities of GST and SOD were assessed in the liver of the rats (Figure 1, a-f). Gatifloxacin resulted in a dose-dependent significant (p<0.05) reduction in the levels of hepatic GSH and H_2S with a concomitant significant (p<0.05) dose-dependent increase in the levels of TBARS and NO. Although the activity of SOD also followed a dose-dependent significant (p<0,05) decrease only 40 mg/kg and 80 mg/kg resulted in significant (p<0.05) decrease in GST activity.

3.2 Gatifloxacin modulated the expression of genes involved in epigenetic regulations in rat liver

The level of expression of *Dnmt1* was significantly (p<0.05) increased only in the liver of rats treated with 80 mg/kg (Figure 2a). However, gatifloxacin administration resulted in significant (p<0.05) decrease in the expression of *Hdac5* at 10 mg/kg; though, none of the higher dosages significantly altered its expression (Figure 2b). While a dose-dependent significant (p<0.05) increase was observed in level of expression of *Ehmt2* and *Suv39h1*, only 80 mg/kg significantly

(p<0.05) increased the level of expression of *Eid3* and *Prdm2* (Figure 2, c-f).

3.3 Gatifloxacin modulated the expression of genes involved in apoptosis in rat liver

The expression of *Bcl2l1*, *Casp3*, *Casp8* and *Casp9* are depicted in figure 3 (a-d). There was a significant ($p < 0.05$) increase in the expression of *Bcl2l1* in the liver of rats treated with 20 mg/kg gatifloxacin with a further increase in group treated with 80 mg/kg. Although a significant ($p < 0.05$) dose-dependent increase was observed in the levels of expression of *Casp8* and *Casp9*, the increase in the dosage of gatifloxacin beyond 10 mg/kg had no significant ($p > 0.05$) effect on the level of expression of *Casp3*.

Table 1: List of genes studied and the sequences of Gene Specific Primers

| Gene Code	Gene name	Primer Sequence (5'->3')	Template
Prdm2	PR/SET domain 2 methyltransferase	Forward: CGGATTGGTGTCTGGGCTAC	NM_001077648.1
		Reverse: AAGCCAAAGGCCTCTCATCC	
Hdac5	Histone deacetylase 5	Forward: TTGCTTGGGCCCTATGACAG	NM_053450.1
		Reverse: GGTGAGGTGCGAGTTGGTAA	
Eid3	EP300 interacting inhibitor of differentiation 3	Forward: CGCCCAGTTTCTGGTTTTGG	NM_001044304.1
		Reverse: TTGGCTCGAGAATTGGCAGT	
Suv39h1	Suppressor of variegation 3-9 homolog 1	Forward: GGCGACTCTAGGTTGCAGTG	NM_001106956.1
		Reverse: GGCCTTCTGCACCAGGTAAT	
Ehmt2	Euchromatic histone lysine methyltransferase 2	Forward: GTCCCTTGTCTCCCCTCCC	NM_212463.1
		Reverse: AGAGCCACTCCTGTCTGACT	
Dnmt1	DNA methyltransferase 1	Forward: AGAACGGAACACTCTCTCACTCA	NM_053354.3
		Reverse: AAGCTTCAATCATGGTCTCACTGTC	
Bcl2l1	Bcl-2-like 1	Forward: TTTTGCTGAGTTACCGGCGA	NM_001033672.1
		Reverse: GCCACAAGGGTAGCCAGAAT	
Casp3	Caspase 3	Forward: GAGCTTGGAACGCGAAGAAA	NM_012922.2
		Reverse: TAACCGGGTGCGGTAGAGTA	
Casp8	Caspase 8	Forward: AGAGAAGCAGCCTATGCCAC	NM_022277.1
		Reverse: CCCCGAGGTTTGCTCTTCAT	
Casp9	Caspase 9	Forward: GCGCGACATGATCGAGGATA	NM_031632.1
		Reverse: TCTCCATCAAAGCCGTGACC	
β-ACTIN	Actin, Beta	Forward: GTCAGGTCATCACTATCGGCAAT	NM_031144.3
		Reverse: AGAGGTCTTTACGGATGTCAACGT	

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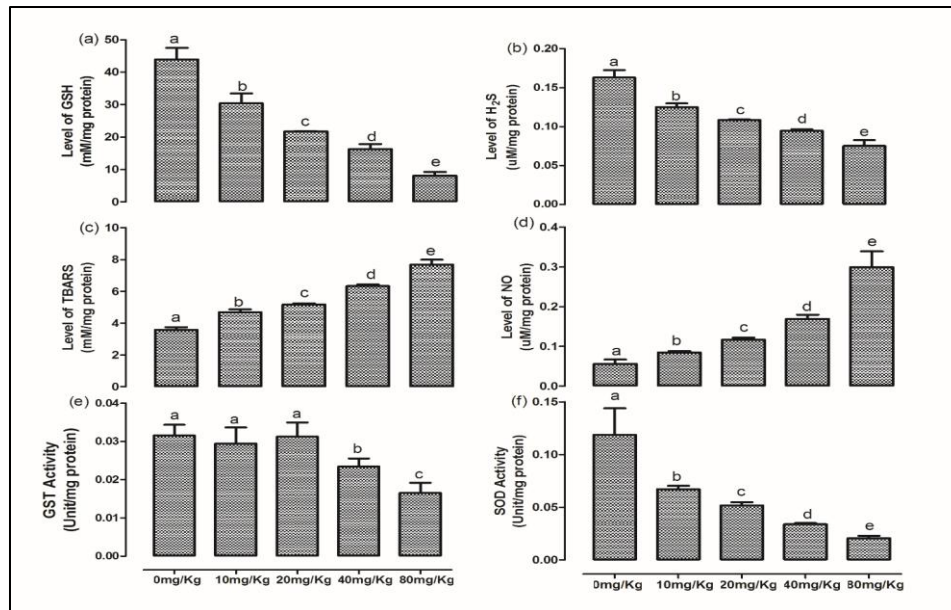
Figure 1 (a-f): Effects of gatifloxacin on biomarkers of oxidative stress in rat liver. (a) The levels of liver reduced glutathione, (b) the levels of liver hydrogen sulfide (c) the level of liver thiobaribituric acid reactive substances, (d) the level of liver nitric oxide, (e) the activity of liver gluthathione-s-transferase and (f) the activity of superoxide dismutase.
Bars represent mean ± SEM (n=6). Bars with different statistical markers are significantly different at p<0.05.
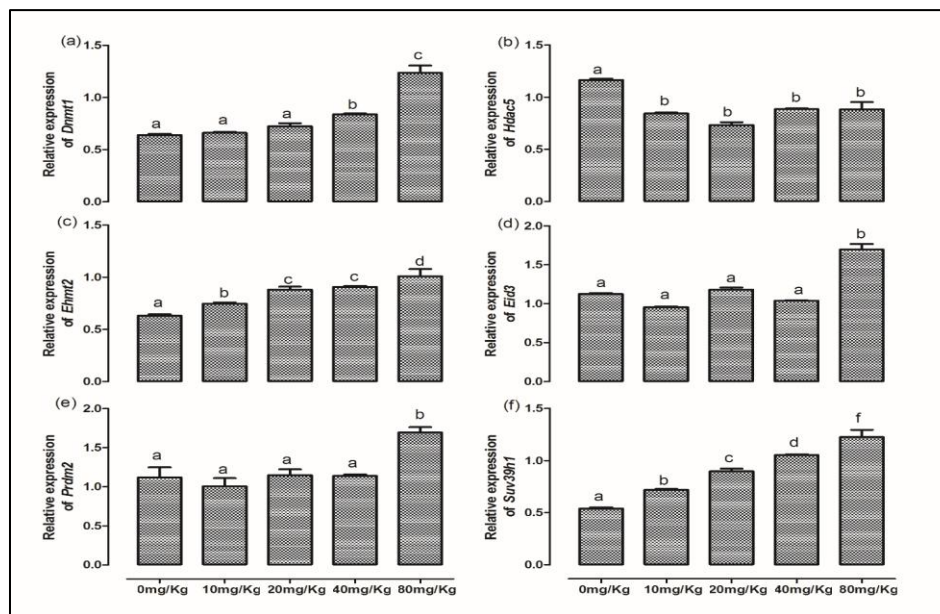

Figure 2 (a-f): Effects of gatifloxacin on genes involved in epigenetic regulations. (a) The levels of expression of hepatic _Dnmt1_, (b) the levels of expression of hepatic _Hdac5_ (c) the levels of expression of hepatic _Ehmt2_, (d) the levels of expression of hepatic _Eid3_, (e) the levels of expression of hepatic _Prdm2_ and (f) the levels of expression of hepatic _Suv39h1_.
Bars represent mean ± SEM (n=6). Bars with different statistical markers are significantly different at p<0.05.
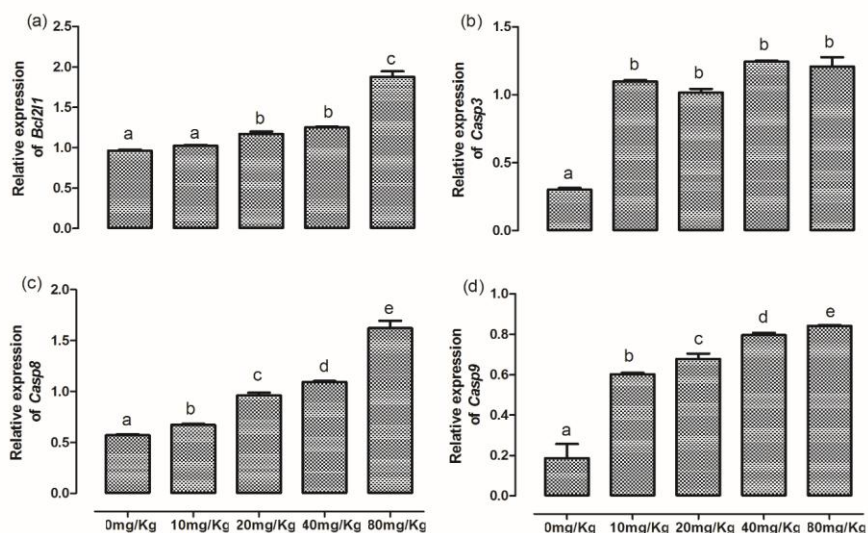
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Figure 3 (a-d): Effects of gatifloxacin on genes involved in apoptosis. (a) The levels of expression of hepatic _Bcl2l1_, (b) the levels of expression of hepatic caspase 3 (c) the levels of expression of hepatic caspase 8 and (d) the levels of expression of hepatic caspase 9.
Bars represent mean ± SEM (n=6). Bars with different statistical markers are significantly different at $p<0.05$.

3. Discussion

The ability of gatifloxacin to induce hepatic oxidative stress in rats was investigated by analyzing the levels of TBARS, H_2S, NO and GSH as well as the activities of GST and SOD. Our findings showed that gatifloxacin induced oxidative stress in a dose-depend manner. Kumbhar et al [6] reported a similar dose-dependent induction of oxidative stress in rabbits treated with gatifloxacin. In this study, as well as that of Talla and Veerareddy [1], oxidative stress was characterized by decreased glutathione and hydrogen sulfide levels, and activities of GST and SOD with an associated increase in the level of nitric oxide and TBARS. As part of their bactericidal mechanism, fluoroquinolones trigger the transcriptional activation of iron transport genes and enhance the Fenton reaction resulting in the production of ROS [22]. Also, a recent report by Pan et al [23] showed that fluoroquinolones could decrease SOD activity by forming a complex through hydrogen bonds and van der Waals forces resulting in inhibition and subsequent oxidative stress.

Nitric oxide and hydrogen sulfide are biological messengers that contribute to many physiological processes and play important roles in response to xenobiotics [24]. Although NO is a potent antioxidant that rapidly neutralizes superoxide anion, it is subsequently converted to prooxidant and its biphasic action of protection at low concentrations and oxidative killing of cells at high concentration has been reported [25]. On the other hand, H_2S regulates GSH biosynthesis from GSSG [26]. The depletion of hepatic H_2S metabolism has been implicated in the pathogenesis of many liver diseases [26] and our findings suggests that it could also play a role in the pathogenesis of gatifloxacin-induced liver damage.

The interaction between fluoroquinolones and iron also alters the epigenetic signature of the cell through inhibition of dioxygenases that require iron as a co-factor [27]. Such epigenetic alterations may include DNA methylation and histone modifications. Our findings showed that gatifloxacin altered the expressions of _Dnmt1, Hdac5, Prdm2, Eid3, Suv39h1_ and

Ehmt2. The *Dnmt1* is responsible for methylating cytosine residues of DNA and aberrant methylation patterns, resulting from increased *Dnmt1* expression, are associated with etiology of certain diseases, especially liver disorders [28, 29]. On the other hand, histone modification could occur via methylation or deacetylation. Histone methylation is achieved by an array of methyltransferases which include *Prdm2, Eid3, Suv39h1* and *Ehmt2* [30, 31] that methylate the histone lysine residues. Therefore, these methyltransferases are key components in cellular processes, and alteration in their expression is associated with pathogenesis [31]. Histone deacetylase is another protein involved in this mechanism and it is responsible for deacetylation of lysine residues on the N-terminal of core histones [32, 33]. Previous studies have reported certain quinolones to inhibit this enzyme [32] and such inhibition or decrease in expression of *Hdac5* has been reported to induce growth arrest, differentiation, and/or apoptotic cell death [33, 34].

Interestingly, the induction of apoptosis by certain fluoroquinolones has been reported [35, 36]. In this present study, gatifloxacin resulted in dose-dependent upregulation of *Bcl2l1*and caspases 3, 8 and 9. Previous studies have reported increase in expression of these proteins by a novel bis-fluoroquinolone compound [37], levofloxacin [38] and ciprofloxacin [39].

4. Conclusion

Our findings therefore demonstrated that gatifloxacin-induced oxidative stress is associated with alterations in expression of epigenetic and proapoptotic genes. These alterations in gene expression could be part of the underlining mechanisms resulting in hepatotoxicity of gatifloxacin.

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