Alterations of Genes Involved in Apoptosis and Epigenetic Modulation Associated with Gatifloxacin-Induced Oxidative Stress in Rat Liver

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ABSTRACT

Gatifloxacin is a fourth-generation fluoroquinolone that induces oxidative stress in the liver. Hence, this study investigated the alterations in the expression of genes involved in epigenetics and apoptosis associated with gatifloxacin-induced oxidative stress in rat liver. To achieve this, adult rats were exposed to oral doses (10 mg/kg, 20 mg/kg, 40 mg/kg and 80 mg/kg) of gatifloxacin for five days. Thereafter, 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 reverse transcription polymerase chain reaction technique. The results showed that the hepatic oxidative stress was associated with increase in the expression of proapoptotic genes. Also, gatifloxacin resulted in significant ($p < 0.05$) upregulation of genes involved in DNA and histone methylation. These alterations observed at the lowest dose of 10 mg/kg showed that gatifloxacin exposure could induce hepatic apoptosis and epigenetic changes.

Keywords: Gatifloxacin; Oxidative Stress; Epigenetics; Apoptosis.

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 et al [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 [6-8]. 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 [9, 10]. Epigenetic modifications are modifications affecting the expression of DNA without affecting the DNA sequence. These modifications include DNA methylation and histone modifications [11, 12]. 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 [13].

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.

MATERIAL AND METHODS

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 of analytical standard and purchased from Sigma-Aldrich.

Experimental animals and procedure

Twenty-five (25) inbred male Wistar rats (130±30 g) were used for this research. The animals were maintained on standard 12-h light and dark cycles and granted access to water and feed, *ad libitum*. The animals were allowed to acclimatize for three weeks before commencement of the experiment. The experiment was approved by the Covenant University Ethical Committee (CU/BIOSCRECU/BIO/2016/004) and carried out according to the guidelines of the committee. Thereafter, the animals were randomly allotted into five (5) experimental groups after the initial 2 weeks of acclimatization. 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. Twenty-four (24) hours after the last dosage, the rats were anaesthesized under light ether and sacrificed. The liver was excised immediately and its portion for oxidative stress assays were processed appropriately[14], while other portions were cryopreserved in RNAhold® for RNA analysis.

Biochemical analysis

The level of lipid peroxidation was quantified by assessing the concentration of thiobarbituric acid reactive substances (TBARS) as described by Buege and Aust[15]. Glutathione-S-transferase's activity was assayed using as described by Habig[16], by measuring the rate of conjugation of glutathione and 1-Chloro-2,4-dinitrobenzene at 340 nm. Superoxide dismutase's activity was determined as described by Marklund and Marklund,[17] by measuring the rate of autooxidation of pyrogallol at 420 nm. The level of reduced glutathione (GSH) concentration was quantified according to the method of Ellman[18]. Nitric oxide (NO) concentration was assayed as described by Yucel *et al.,*[19] using the Griess reaction method. The Lowry method was used for the determination of protein concentration as described by Gallagher and Desjardins[20].

The tissue level of hydrogen sulfide (H_2S) was assayed using the methylene blue formation method as described Shen et al.[21]. Briefly, 75 ¼L of liver homogenate was mixed with 250 µL Zn acetate (1%) and 450 µL distilled water for 10 min at room

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 Reverse: AAGCCAAAGGCCTCTCATCC	NM_001077648.1
Hdac5	Histone deacetylase 5	Forward: TTGCTTGGGCCCTATGACAG Reverse: GGTGAGGTGCGAGTTGGTAA	NM_053450.1
Eid3	EP300 interacting inhibitor of differentiation 3	Forward: CGCCCAGTTTCTGGTTTGG Reverse: TTGGCTCGAGAATTGGCAGT	NM_001044304.1
Suv39h1	Suppressor of variegation 3-9 homolog 1	Forward: GGCGACTCTAGGTTGCAGTG Reverse: GGCCTTCTGCACCAGGTAAT	NM_001106956.1
Ehmt2	Euchromatic histone lysine methyltransferase 2	Forward: GTCCCTTGTCTCCCCTCCC Reverse: AGAGCCACTCCTGTCTGACT	NM_212463.1
Dnmt1	DNA methyltransferase 1	Forward: AGAACGGAACACTCTCTCTCACTCA Reverse: AAGCTTCAATCATGGTCTCACTGTC	NM_053354.3
Bcl2l1	Bcl-2-like 1	Forward: TTTTGCTGAGTTACCGGCGA Reverse: GCCACAAGGGTAGCCAGAAT	NM_001033672.1
Casp3	Caspase 3	Forward: GAGCTTGGAACGCGAAGAAA Reverse: TAACCGGGTGCGGTAGAGTA	NM_012922.2
Casp8	Caspase 8	Forward: AGAGAAGCAGCCTATGCCAC Reverse: CCCCGAGGTTTGCTCTTCAT	NM_022277.1
Casp9	Caspase 9	Forward: GCGCGACATGATCGAGGATA Reverse: TCTCCATCAAAAGCCGTGACC	NM_031632.1
[2]ACTIN	Actin, Beta	Forward: GTCAGGTCATCACTATCGGCAAT Reverse: AGAGGTCTTTACGGATGTCAACGT	NM_031144.3

temperature. TCA (10%; 250 µL) was then added, centrifuged at 14,000 g for 10 min. The supernatant was reacted with N,N-dimethyl-p-phenylenediamine sulfate (20 mM/L; 133 ¼L) and FeCl$_3$ (30 mM/L; 133 ¼L) and the absorbance was read at 670 nm after 20 min.

Gene expression analysis

The expression level of certain apoptotic, DNA methylating and chromatin modifying genes

(Table 1) were quantified using relative reverse transcriptase polymerase chain reaction (RT-PCR) techniques as described by Chaudhry[22],with appropriate modifications. In brief, RNA was extracted from the liver using Aidlab® EASYspin Plus® kit following the manufacturer's guideline. About 500 ng of RNA was used for the RT-PCR using the Transgen® *EasyScript®* one-step RT-PCR

reagent. Briefly, the cDNA synthesis was carried out at 45°C for 30 minutes. This was followed by 35 cycles of PCR amplification, using gene specific primers (GSP) (Table 1), in a C1000 Touch™ Thermal Cycler (BioRad, CA, USA). The cycles consisted of 94°C for 30s, 5min at the annealing temperature of GSP and 1min at 72°C.

The level of transcription of the genes relative to ²-actin was quantified using Image J® software[23, 24].

Statistical analysis

Data were expressed as mean ± SEM and analysis of variance was carried out to test for the level of homogeneity at p Â 0.05 among the groups. Heterogeneous groups were subjected to Duncan's multiple range post hoc test.
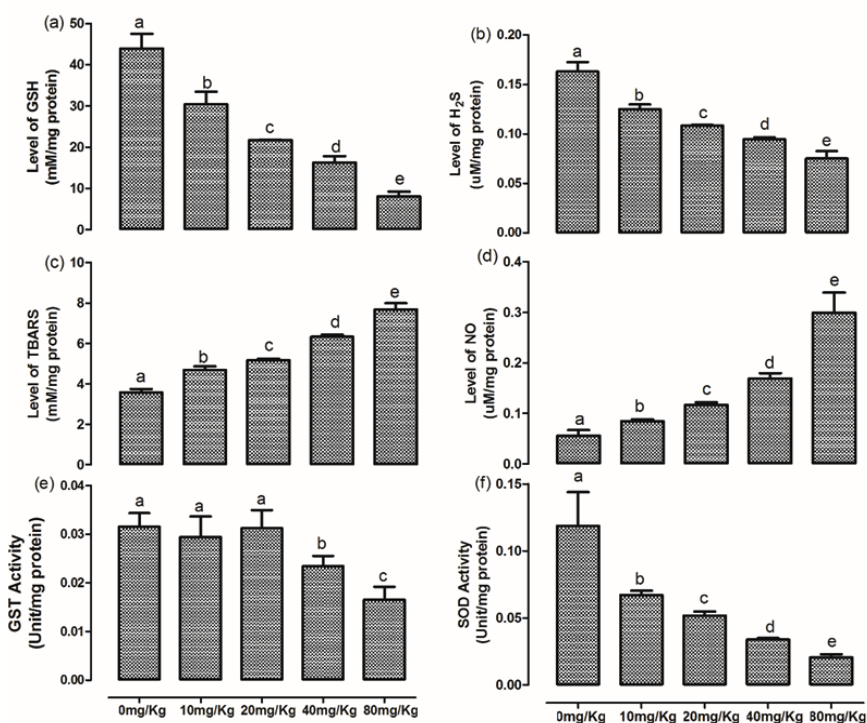
Bars represent mean ± SEM (n=6). Bars with different statistical markers are significantly different at p<0.05.

Fig. 1: (a-f): Effects of gatifloxacin on oxidative stress in rat liver. (a) levels of liver reduced glutathione, (b) levels of liver hydrogen sulfide (c) 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

RESULTS

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.

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).
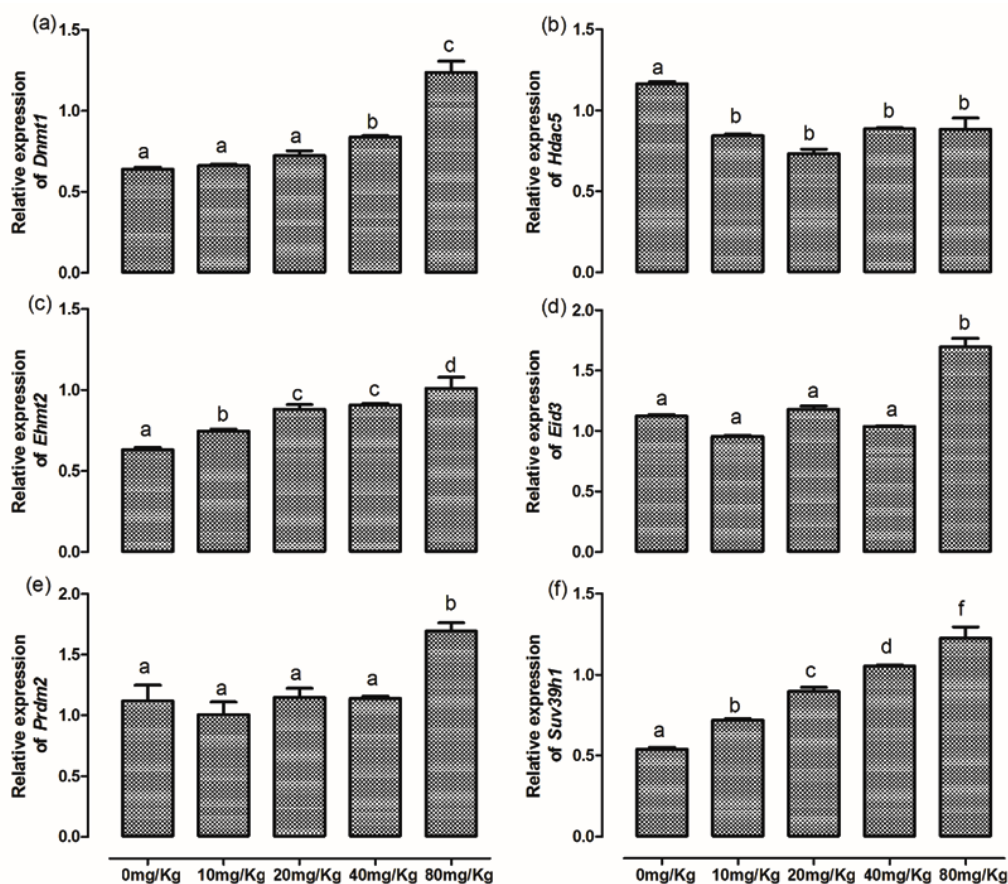
Bars represent mean ± SEM (n=6). Bars with different statistical markers are significantly different at p<0.05.

Fig. 2: (a-f): Effects of gatifloxacin on genes involved in epigenetic regulations. (a) The levels of expression of Dnmt1 in the liver, (b) the levels of expression of Hdac5 in the liver, (c) the levels of expression of Ehmt2 in the liver, (d) the levels of expression of Eid3 in the liver, (e) the levels of expression of Prdm2 in the liver and (f) the levels of expression of Suv39h1 in the liver

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 expression of *Casp3*.

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 GSH and H_2S 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 [25]. Also, a recent report by Pan et al[26] 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 (NO) and H_2S are biological messengers that contribute to many physiological processes and play important roles in response to xenobiotics [27]. 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
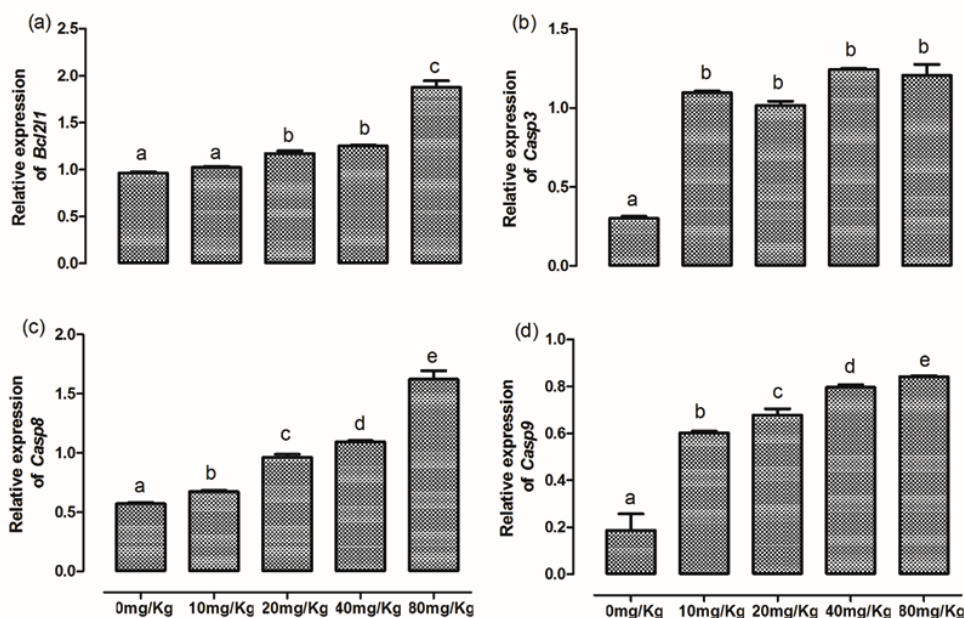
Bars represent mean ± SEM (n=6). Bars with different statistical markers are significantly different at p<0.05.

Fig. 3: (a-d): Effects of gatifloxacin on genes involved in apoptosis. (a) The levels of expression of Bcl2l1 in the liver, (b) the levels of expression of caspase 3 in the liver, (c) the levels of expression of caspase 8 in the liver and (d) the levels of expression of caspase 9 in the liver

been reported [28]. On the other hand, H_2S regulates GSH biosynthesis from GSSG[29]. The depletion of hepatic H_2S metabolism has been implicated in the pathogenesis of many liver diseases[29] and our findings suggests that it could also be involved 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[30]. 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[31, 32]. 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* [33, 34] that methylate the histone lysine residues. Therefore, these methyltransferases are key components in cellular processes, and alteration in their expression is associated with pathogenesis

[34]. Histone deacetylase is another protein involved in this mechanism and it deacetylates the lysine residues on the N-terminal of core histones[35, 36]. Previous studies have reported certain quinolones to inhibit this enzyme [35] and such inhibition or decrease in expression of *Hdac5* has been reported to induce growth arrest, differentiation, and/or apoptotic cell death [36, 37].

Interestingly, the induction of apoptosis by certain fluoroquinolones has been reported [38, 39]. In this present study, gatifloxacin administration resulted in a 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 [40], levofloxacin [41] and ciprofloxacin [42].

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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