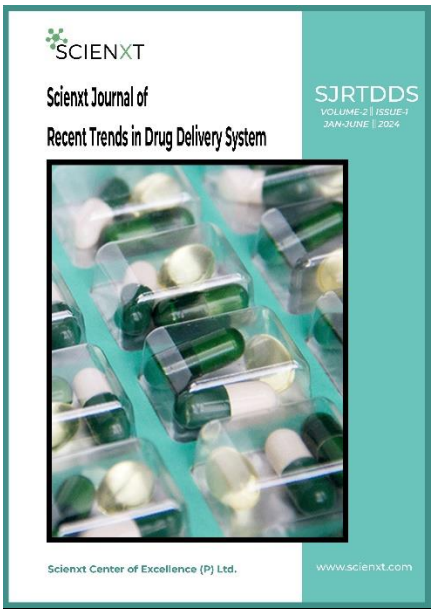

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Alterations of apoptotic and epigenetic genes associated with gatifloxacin-induced oxidative stress in rat liver

[1]Solomon Oladapo Rotimi, [2]Iyanuoluwa Temitayo, [3]Olugbemi and [4]OluwakemiAnuoluwapo Rotimi

Biochemistry Unit and Molecular Biology Research Laboratory, Department of BiologicalSciences, Covenant University, Ota, Ogun State, Nigeria.

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 andtrigger 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-generationfluoroquinolone 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. Fluoroquinolones, including gatifloxacin, have been reported to produce several sideeffects including hepatotoxicity, joint defects and phototoxicity with complications like liver damage, purpura and dysglycemia. In particular, gatifloxacin has been reported to induce fulminant hepatic failure [3]. Olayinka, Ore 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 sideeffects like phototoxicity, cartilage damage and liver damage have been linked to the generation of reactive oxygen species (ROS) leading to oxidative stress. Fluoroquinolonespenetrate neutrophils and enhance their antimicrobial activity by generating ROS. Although studies have shown the potential of gatifloxacin to induce oxidative stress, there isdearth of information on whether the induced oxidative stress alters the expression of genes involved in oxidative DNA damage/repair.

Evidences are now emerging that oxidativestress is accompanied with changes in epigenetic signature of the DNA in the liver and that xenobiotics can modulate thesechanges. Epigenetic modifications are modifications affecting the expression ofDNA without affecting the DNA sequence.These modifications include DNA methylation and histone modifications. 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 canalter pluripotency by interfering with histone modification signature.

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 inrat liver.

2. Material and methods:

2.1. Chemicals and reagents:

Gatifloxacin was obtained from Sigma- Aldrich, St. Louis, MO. EASYspin Plus® wasobtained from Aidlab Biotechnologies Co., Ltd, Beijing, China while RNAhold® and *EasyScript*® one-step RT-PCR kit wasobtained 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 tostandard 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 while portions of the liver were kept in RNAhold® and stored at -80 ^{o}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. Glutathione-S-transferase's activity was determined according to the method of Habig. Superoxide dismutase's activity was determined according to the method of Marklund and Marklund. Glutathione concentration was determinedaccording to the method of Ellman. 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 proteinconcentration as described by Gallagher and Desjardins.

The tissue level of H_2S was assayed using the methylene blue formation method as described Shen et al. 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,with appropriate modifications. In brief, RNA was extracted from the liver using Aidlab® EASYspin Plus® kit according tothe 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 themanufacturer's instructions. Samples weresubjected to an initial incubation at 45ºC for 30 minutes for cDNA synthesis, followed by PCR amplification, using gene specific primers (GSP) (Table 1), 94ºC for 5min followed by 40 cycles of 94ºC for 30s, 5minat the annealing temperature of GSP and 1minat 72ºC. All amplifications were carried out in C1000 Touch™ Thermal Cycler (BioRad, CA, USA).

The level of transcription of the genes relativeto β-actin was quantified using Image J® software.

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 levelof homogeneity at $p < 0.05$ among the groups. Duncan's multiple range test was used to separate the heterogeneous groups.

3. 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 (Fig. 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 theactivity 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.

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	
Suv39h 1	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: AGAACGGAACACTCTCTCTCACTCA	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	

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 theliver of rats treated with 80 mg/kg (Figure2a). However, gatifloxacin administration resulted in significant ($p<0.05$) decrease in theexpression 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* (Fig. 2, c-f).

3.3. Gatifloxacin modulated the expression of genes involved in apoptosis inrat 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 treatedwith 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 levelsof expression of *Casp8* and *Casp9*, theincrease in the dosage of gatifloxacin beyond 10 mg/kg had no significant ($p > 0.05$) effect on the level of expression of *Casp3*.

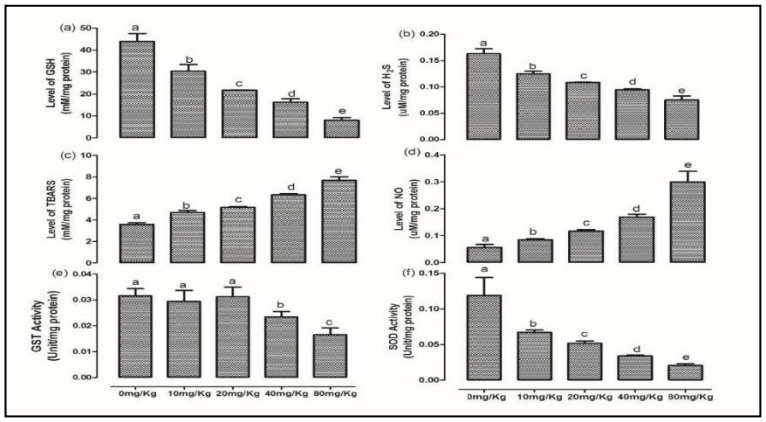

Figure. 1: (a-f): Effects of gatifloxacin on biomarkers of oxidative stress in rat liver

(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 $p<0.05$.

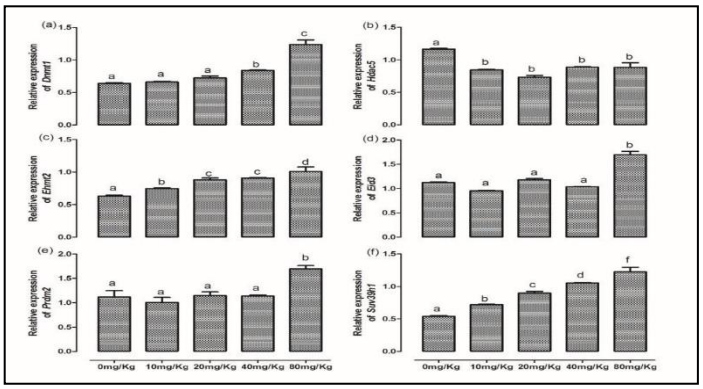

Figure. 2: (a-f): Effects of gatifloxacin on genes involved in epigenetic regulations

(a) The levels of expressionof 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 ofexpression of hepatic *Suv39h1*.

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

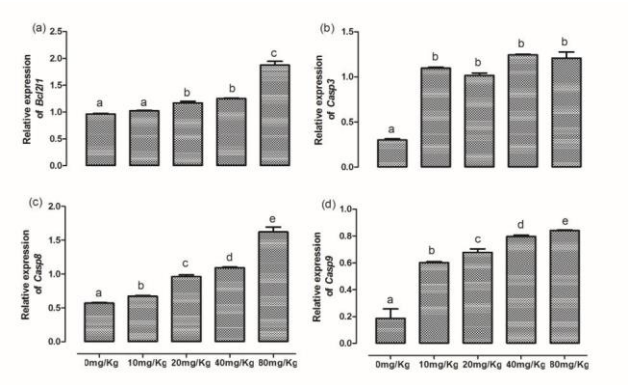

Figure 3 (a-d): Effects of gatifloxacin on genes involved in apoptosis

(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 $p<0.05$.

4. Discussion:

The ability of gatifloxacin to induce hepatic oxidative stress in rats was investigated byanalyzing the levels of TBARS, H_2S, NO and GSH as well as the activities of GST andSOD. 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, oxidative stress was characterized by decreased glutathione and hydrogen sulfidelevels, and activities of GST and SOD with anassociated increase in the level of nitric oxide and TBARS. As part of their bactericidal mechanism, fluoroquinolones trigger thetranscriptional activation of iron transport genes and enhance the Fenton reaction resulting in the production of ROS. Also, a recent report by Pan et al 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 too manyphysiological processes and play important roles in response to xenobiotics. Although NO is a potent antioxidant that rapidlyneutralizes superoxide anion, it issubsequently converted to prooxidant and its biphasic action of protection at low concentrations and oxidative killing of cells athigh concentration has been reported. On the other hand, H_2S regulates GSH biosynthesis from GSSG. The depletion of hepatic H_2S metabolism has been implicated in the pathogenesis of many liver diseases and our findings suggests that it could also play a role in the pathogenesis of gatifloxacin-induced liver damage.

The interaction between fluoroquinolones andiron also alters the epigenetic signature of the cell through inhibition of dioxygenases that require iron as a co-factor. Such epigenetic alterations may include DNA methylation andhistone modifications. Our findings showedthat gatifloxacin altered the expressions of *Dnmt1, Hdac5, Prdm2, Eid3, Suv39h1* and *Ehmt2*. The *Dnmt1* is responsible formethylating cytosine residues of DNA and aberrant methylation patterns, resulting from increased *Dnmt1* expression, are associated with etiology of certain diseases, especially liver disorders. 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* that methylate the histone lysine residues. Therefore, these methyltransferases are key components in cellular processes, and alteration in their expression is associated with pathogenesis. 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. Previous studies have reported certain quinolones to inhibit this enzyme and such inhibition or decrease in expression of *Hdac5* has been reported to induce growth arrest, differentiation, and/or apoptotic cell death. Interestingly, the induction of apoptosis bycertain fluoroquinolones has been reported. In this present study, gatifloxacin resulted in dose-dependent upregulation of *Bcl2l1*and caspases 3, 8 and 9. Previous studies havereported increase in expression of these proteins by a novel bis-fluoroquinolone compound, levofloxacin and ciprofloxacin.

5. Conclusion:

Our findings therefore demonstrated that gatifloxacin-induced oxidative stress is associated with alterations in expression ofepigenetic and proapoptotic genes. Thesealterations in gene expression could be part ofthe underlying mechanisms resulting in hepatotoxicity of gatifloxacin.

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