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Comparison of hepatoprotective effects of clofibrate and its novel siliconized analogue in isolated rat hepatocytes

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ABSTRACT

Background and the purpose of the study: Peroxisome proliferator ligands have been found to have a hepatoprotective effect against induced injuries in hepatotoxicants. FeIII:8HQ induces oxidative stress in freshly isolated cells. The hepatoprotective effects of clofibrate and its novel siliconized analog (silafibrate) against the FeIII:8HQ complex are compared here for the first time. Methods: A siliconized analog of clofibrate synthesized by replacement of the chlorine atom in the phenoxy ring with trimethylsilyland ethyl2-methyl-2-(4-(trimethylsilyl)phenoxy)propionate was prepared. Hepatocytes were obtained from male rats by a two-step collagenase perfusion. The viability of isolated hepatocytes was evaluated by Trypan blue exclusion method. Levels of reactive oxygen species (ROS) were measured with the fluorescent probes2',7'-dichlorofluorescein diacetate (DCFHDA). Mitochondrial membrane potential was measured by using Rhodamine 123 fluorescence. Results: Incubation of hepatocytes with low to moderately toxic doses of silafibrate (200, 250, 400, and 500 μM) for 3 hours did not evoke a notable toxic response in three time-repeated experiments. However, higher doses (1, 2mM) have significant toxicity in Trypan blue exclusion cell viability experiments. Mitochondrial membrane potential decrease was prevented by pretreatment of hepatocytes with clofibrate and/or silafibrate, 20 minutes before adding FeIII:8HQ complexes (I, II). 100 μM clofibrate protected hepatocytes against FeIII: 8HQ induced ROS production, whereas silafibrate with 100, 200, and 400 μM strongly inhibited ROS production. Conclusion: These results demonstrate that fibrates have an in vitro hepatoprotective effect against oxidative stress. Silafibrate, the novel analog, has a better effect in protection against oxidative stress in comparison with clofibrate.

Keywords: Hepatocytes, Clofibrate, Silafibrate, Oxidative stress.
Abbreviations:ANOVA, analysis of variance; APX, ascorbate peroxidase; BSA, bovine serum albumin; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; FeIII:HQ, ferric 8-hydroxyquinoline; H_2O_2, hydrogen peroxide; HDL, High-density lipoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LDL, Low-density lipoprotein; MDAR, monodehydroascorbate reductase; MPTP, mitochondrial permeability transition pore ; PPAR, peroxisome proliferator activated receptor; ROS, reactive oxygen species; rpm, rotations per minute; SD, standard deviation; SOD, superoxide dismutase; TBARS, 2-thiobarbituric acid-reactive substances; $\Delta\Psi_m$, percentage of mitochondrial membrane potential decline.
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INTRODUCTION

Clofibrate is a synthetic agonist of peroxisome proliferator activated receptor-*α* (PPAR-*α*). PPARs are a nuclear receptor super family naturally activated by a large variety of fatty acids and fatty acid metabolites, such as hydroxylated eicosanoids, prostaglandins, and leukotrienes, and by many synthetic compounds [41]. These receptors are ligand-dependent transcription factors that are classified as three subtypes known as PPAR-*α*, PPAR-β/δ, and PPAR-γ. Peroxisome proliferator-activated receptors (PPARs) perform diverse roles in metabolic processes, such as H_2O_2-derived respiration, β-oxidation of fatty acids, and cholesterol metabolism [11]. PPAR-α is highly expressed in hepatocytes, cardiomyocytes, enterocytes, and renal proximal tubule cells [17]. PPAR-*α* increases hepatic peroxisome volume and density, or peroxisome proliferation. Observations show that fenofibrate may boost endothelial function, reveal antioxidant and anti-inflammatory effects, attenuate thrombotic process, and decrease serum uric acid levels [48]. It has been clearly demonstrated that treatment with peroxisome proliferative ligands such as clofibrate could prevent chemically induced oxidative injuries [18,49]. Nafenopin, a PPARs activator, weakens hydrogen peroxide toxicity in cultured rat primary hepatocytes [34]. Pretreatment of mice with fibrates alters the generation of thiobarbituric acid reactive substances (TBARS) induced by acute doses of tri- and dichloroacetic acid [19].

Oxidative stress is caused by an imbalance between the productions of reactive oxygen species (ROS) and the biological system's capability to rapidly neutralize the reactive intermediates [12]. ROS have a major effect on oxidation of low density lipoproteins (LDLs) and afterward in the formation of atherogenesis. Various oxidative stresses such as elevation of ROS production and/or impaired antioxidant defense are likely to result in excessive peroxidation of polyunsaturated fatty acids contained in LDL particles and, therefore, may accelerate atherogenesis. Previous studies concerning the effect of fibrates on oxidative stresses are extremely controversial. Some of them reported antioxidant activity of these drugs [7, 15, 26, 30, 39]. whereas other studies demonstrated no effect [5, 22, 33] or even prooxidant properties of fibrates [8, 24, 40].

Investigation for effective and safe lipid lowering agents has engaged the interests of medicinal chemists, biochemists, pharmacologists, and clinicians. The use of organosilicon chemistry in drug design has been previously reviewed [20, 36, 38, 42, 50]. In general, sila-replacement may affect the chemical and physicochemical traits and may alter the biological properties; for example, modified bond lengths and angles may change the molecule's interaction with a receptor and, thus, the pharmacological selectivity and/or potency [44]. Recently, silicon switches of marketed drugs have been reviewed [36]. Siliconized analog of prevalent drugs modify the geometric and electronic aspects and, therefore, the size, shape, conformational behavior, chemical reactivity, and lipophilicity of the molecule. This might, in turn, change the interaction with a receptor and, thus, alter the pharmacodynamics of the drug. The metabolism of the drug may change and, therefore, also metabolism-related toxicity [44]. Examples of silicon switches are sila-haloperidol, sila-venlafaxine, sila-fexofenadine, and disila-bexarotene, which are studied in different in vitro systems [36]. Fibric acid derivatives (fibrates) represent an important class of lipid modifying agents. In the search for analogs of clofibrate, a siliconized analog (silafibrate) was synthesized, whereby the chlorine atom in the phenoxy ring was replaced by silicon, and ethyl2-methyl-2-(4-(trimethylsilyl)phenoxy)propionate was prepared (Figure 1) . Clofibrate, developed in 1965, the first fibrate drug, is no longer recommended as a lipid-lowering agent [18].

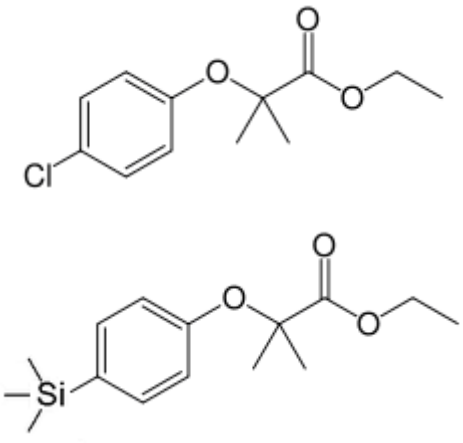

Figure 1. Chemical structures of clofibrate (upper panel) and Silafibrate (lower panel)

Freshly isolated hepatocytes in suspension contain a complete complement of drug metabolizing enzymes, remain viable for several hours, and possess relevant drug transport proteins [16, 37]. As a minor part of an extensive study on silafibrate, including synthesis and pharmacological properties, its effects on freshly isolated rat hepatocytes were tentatively identified.

In this study, we have compared the effects of clofibrate with those of silafibrate, in freshly isolated hepatocytes exposed to oxidative stress induced by FeIII-8HQ toxic complex.

MATERIALS AND METHODS

Chemicals

Clofibrate was gifted by Zahravi Pharmaceutical Inc (Tabriz - Iran). A siliconized analog of clofibrate that is here called *Silafibrate* was synthesed in Chemistry and Chemical Engineering Research Center of Iran (Tehran – Iran).
Bovine serum albumin (BSA), collagenase A (from Clostridium histolyticum) and N-(2-hydroxyethyl) piperazine-N0-(2-ethanesulfonic acid) (HEPES) were obtained from Roche diagnostics (Indianapolis, USA), 2′,7′-dichlorofluorescein diacetate (DCFHDA) and Rhodamine 123, from FLUKA. Heparin sodium salt grade 1-A, Trypan Blue (0.2%, w/v), Methanol, MgSO4, and other buffer salts were obtained from Merck (Germany). All other chemicals used were of the highest analytical grade commercially available.

Animals

Male Sprague-Dawley rats of about 250-280 g body weight were used in this study for hepatocyte preparation. The animals obtained from Animal House of Tabriz University of Medical Sciences were kept at a controlled ambient temperature of 20°C-25 °C with 40 ± 10% relative humidity and with a 12/12-h light/dark cycle condition. All animals were allowed to access standard laboratory chow and watering ad libitum. Rats were acclimatized 1 week before the experiments. Anesthesia was induced with pentobarbital (50 mg/kg i.p.). All procedures on animals in this study followed the guidelines approved by the Animal Care and Use Committee of Tabriz University of Medical Sciences. The ethical standards were based on ‘‘European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes” Acts of 1986, and the ‘‘Guiding Principles in the Use of Animals in Toxicology,” adopted by the Society of Toxicology in 1989, for the acceptable use of experimental animals.

Isolation and incubation of hepatocytes

Hepatocytes were obtained from male Sprague-Dawley rats by a two-step collagenase perfusion, as previously described [25]. The first step involves the perfusion of a calcium-free buffer. The second step is circulation of a calcium-supplemented buffer containing collagenase. The initial perfusion facilitates desmosomal cleavage and further dispersion of liver cells. The addition of Ca^{2+} to the enzyme solution ensures adequate collagenase activity. After isolation, the cells were suspended at a density of 10^6 cells/ml in Krebs-Henseleit buffer (pH=7.4) containing 12.5 mM HEPES and incubated under an atmosphere of 95% O2 and 5% CO2 in continuous rotating round bottomed 50 ml flasks at 37 °C. Each flask contained 10 ml of hepatocyte suspension. Hepatocytes were preincubated for 20 min before the addition of chemicals. Stock solutions of all chemicals were freshly prepared before use.

Cell viability

The viability of isolated hepatocytes was evaluated by Trypan blue (0.2% w/v) exclusion method from the intactness of the plasma membrane [25]. Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period and were combined with 0.2% trypan blue in a test tube, and the mixture was counted for cells using a hemocytometer. The hepatocytes used in this study were at least 85-90% viable immediately after isolation.

Determination of reactive oxygen species

Production of intracellular reactive oxygen species (ROS) was monitored by the fluorescence emission of 2′,7′-dichlorofluorescein diacetate (DCFHDA). To determine the rate of ROS generation induced by silafibrate and clofibrate, DCFHDA was added to the hepatocyte incubation. It diffuses hepatocyte cells membrane and enzymatically becomes hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescin (DCFH). In the presence of ROS, it oxidized to highly fluorescent dichlorofluorescein (DCF), which effluxes the cell [31]. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrofluorometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were shown as fluorescent intensity per 10^6 cells [13].

Mitochondrial membrane potential assay ($\Delta\psi m$)

Mitochondrial membrane potential of the cells was evaluated by monitoring uptake of the cationic fluorescent dye, rhodamine123 [3]. The uptake and retention of the rhodamine 123 in hepatocytes has been used for the measurement of mitochondrial membrane potential. Selective accumulation of rhodamine 123 in active mitochondria by charge-

facilitated diffusion is the main principle of this assay. Isolated cells were extracted, and then resuspended in original media containing 1 μM Rhodamine123. After 10 min. of incubation, the cells were centrifuged, and the supernatant was measured with a Shimadzu RF-5000U spectrofluorimeter at the excitation wavelength 501nm and the emission wavelength of 530nm. The amount of dye remaining in the supernatant was inversely proportional to the membrane potential of the cells. The capacity of mitochondria to take up the rhodamine 123 was calculated as fluorescence intensity of rhodamine 123 (% of control) [32].

Incubation with cytotoxic complex

Incubation with Fe(III)/8-hydroxyquinoline increases the cellular iron and induces strong hepatocellular injury with morphological features of not only apoptosis, but also necrosis. The iron-induced cell injury is oxygen dependent [47]. Moreover, iron-8HQ was strongly toxic to the cells and inhibited their growth after exposure. It seems that iron-8HQ toxicity is caused by substantial lipid peroxidation and DNA-strand breakage in cultured cells [35]. Iron and hydrogen peroxide are capable of oxidizing a wide range of substrates and causing biological damage. Moreover, loading the hepatocytes with Fe(III)/8-hydroxyquinoline markedly increased the H_2O_2 enhanced cytotoxicity, suggesting that a Fenton system (H_2O_2/FeIII) leads to a toxic product. Cytotoxic complex I is made by a combination of 8-hydroxy- quinoline(12.5μmol) + Fe (1.5 μmol), and complex II has 8-hydroxy-quinoline(25μmol) + Fe (3 μmol) in suspended cells solution.

The reaction, referred to as the *Fenton reaction*, is complex and capable of generating both hydroxyl radicals and higher oxidation states of the iron [4].

Statistical analysis

Statistical comparisons were carried out using a one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test for multiple comparisons in order to determine statistical significance ($p < 0.05$ or less) between treatments and control groups. Results represent the mean ± SD of at least three independent experiments.

RESULTS

Trypan blue exclusion test

At least 80–90% of the control cells were viable after 3 h. As shown in Table 1 incubation of hepatocytes with low to moderately toxic doses of silafibrate (200, 250, 400, and 500 μM) for 3 hours did not evoke a notable toxic response in three time-repeated experiments. However, higher doses (1, 2mM) have significant toxicity in Trypan blue exclusion cell viability experiments.

Incubation of freshly isolated hepatocytes with FeIII: HQ I (8-hydroxyquinoline12.5μmol+Fe 1.5 μmol) and FeIII: HQ II (8-hydroxy- quinoline 25μmol+Fe 3μmol) have considerable increasing cytotoxicity in 3 hours (Table 2). Pretreatment of hepatocytes 20 minutes with clofibrate and/or its novel analog, silafibrate, before adding cytotoxic complexes (FeIII:8HQ I, II) reduced cell death significantly. As shown in Table 2, the incubation of isolated hepatocytes with Fe(III) ⁄8-hydroxyquinoline complex II induced approximately 50% loss in hepatocyte viability within 2 hrs (LC50), as measured by the Trypan blue exclusion assay under normotensive conditions (37 °C).

Table 1. Effect of silafibrate on freshly isolated rat hepatocytes

Compounds added	Cytotoxicity (% Trypan blue uptake)		
	60 min	120 min	180 min
Control	15 ± 3	17 ± 2	20 ± 3
+ Silafibrate 200 μM	15 ± 2	16 ± 3	18 ± 2
+ Silafibrate 250 μM	15 ± 3	16 ± 3	19 ± 3
+ Silafibrate 400 μM	16 ± 2	18 ± 3	21 ± 4
+ Silafibrate 500 μM	16 ± 3	20 ± 2	24 ± 2
+ Silafibrate 1mM	19 ± 2*	25 ± 2*	28 ± 3*
+ Silafibrate 2mM	23 ± 2*	27 ± 3*	32 ± 2*
+ Clofibrate 200 μM	17 ± 3	21 ± 2	24 ± 2
+ Clofibrate 250 μM	18 ± 2	23 ± 3	25 ± 3
+ Clofibrate 400 μM	21 ± 3*	26 ± 2*	28 ± 3*
+ Clofibrate 500 μM	24 ± 3*	27 ± 2*	31 ± 2*
+ Clofibrate 1mM	27 ± 2*	32 ± 3*	34 ± 2*
+ Clofibrate 2mM	30 ± 2*	35 ± 3*	37 ± 3*

Hepatocytes were incubated in Krebs-Henseleit solution pH 7.4 at 37°C under the atmosphere of 10%O2/5%CO2/N2. The samples were taken at mentioned time intervals and cell death was assessed by trypan exclusion.
Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey's HSD test.
**Significant compared with control ($p < 0.05$).*

Table 2. Clofibrate and/or silafibrate markedly reduced cytotoxicity of FeIII:8HQ in isolated rat hepatocytes

Compounds added	Cytotoxicity (% trypan blue uptake)		
	60 min	120 min	180 min
Control	15 ± 3 a b	17 ± 2 a b	20 ± 3 a b
+ DMSO 40 µL/10ml	23 ± 2*a	25 ± 2*	27 ± 2 a b
+ FeCl 30 µM	20 ± 4 a b	22 ± 3 a b	24 ± 2 a b
+ HQ 20 µM	20 ± 3 a b	21 ± 3 a b	22 ± 2 a b
+FeIII:HQ I	38 ± 3* a	45 ± 2* a	62 ± 2 *a
+ Silafibrate 100 µM	25 ± 2 a c	28 ± 3 a c	29 ± 3 a c
+ Silafibrate 200 µM	23 ± 2 a c	26 ± 2 a c	28 ± 3a c
+ Silafibrate 400 µM	22 ± 3 a	24 ± 2 a	27 ± 3 a
+ Clofibrate 100 µM	32 ± 3 a c	34 ± 2 a c	39 ± 3 a c
+ Clofibrate 200 µM	30 ± 2 a c	36 ± 3 a c	38 ± 2 a c
+ Clofibrate 400 µM	27 ± 2 a	29 ± 3 a	32 ± 2 a
+FeIII:HQ II	44 ± 3 b	58 ± 3b	72 ± 2b
+ Silafibrate 100 µM	26 ± 3 b c	31 ± 2 b c	34 ± 3 b c
+ Silafibrate 200 µM	24 ± 3 b c	27 ± 3 b c	31 ± 3 b c
+ Silafibrate 400 µM	22 ± 3 b c	25 ± 3 b c	28 ± 3 b c
+ Clofibrate 100 µM	36 ± 3 b c	38 ± 2 b c	42 ± 3 b c
+ Clofibrate 200 µM	33 ± 2 b c	35 ± 3 b c	39 ± 4 b c

Hepatocytes were incubated in Krebs-Henseleit solution pH 7.4 at 37°C under the atmosphere of 10%O2/5%CO2/N2. The samples were taken at mentioned time intervals and cell death was assessed by trypan exclusion.
FeIII:HQ I: (8-hydroxy- quinoline12.5µmol+Fe 1.5 µmol).
FeIII:HQ II: (8-hydroxy- quinoline 25µmol+Fe 3µmol)
Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey's HSD test.
**Significant compared with control ($p < 0.05$).*
[a]Significant compared with FeIII:HQI($p < 0.05$).
[b]Significant compared with FeIII:HQ II($p < 0.05$).
[c]Significant compared with the same dose of the analogue ($p < 0.05$).

Table 3. Comparison of the effects of clofibrate and/or silafibrate on the mitochondrial membrane potential ($\Delta\Psi_m$) in FeII:8HQ complex-treated hepatocytes.

Compounds added	Δψm (%)		
	60 min	120 min	180 min
Control	100 ± 4	96 ± 3	92 ± 4
+FeIII:HQ I	79.3 ± 5	75.5 ± 3	47.4 ± 4
+ Silafibrate 100 µM	83.2 ± 4 a c	81.42 ± 5 a c	77.16 ± 5 a c
+ Silafibrate 200 µM	88.44 ± 6 a c	85.02 ± 5 a c	80.33 ± 5 a c
+ Silafibrate 400 µM	92.52 ± 4 a c	91.21 ± 4 a c	84.68 ± 4 a c
+ Clofibrate 100 µM	71.83 ± 4 a c	70.02 ± 5 a c	58.32 ± 5 a c
+ Clofibrate 200 µM	77.81 ± 5 a c	75.95 ± 4 a c	62.14 ± 4 a c
+ Clofibrate 400 µM	80.34 ± 5 a c	77.11 ± 5 a c	68.72 ± 4 a c
+FeIII:HQ II	70.7 ± 4	60.6 ± 5	38.7 ± 5
+ Silafibrate 100 µM	80.81 ± 5 b c	72.69 ± 5 b c	71.38 ± 5 b c
+ Silafibrate 200 µM	85.18 ± 5 b c	80.74 ± 4 b c	76.62 ± 4 b c
+ Silafibrate 400 µM	88.56 ± 4 b c	84.47 ± 4 b c	79.49 ± 5 b c
+ Clofibrate 100 µM	74.53 ± 5 b c	70.32 ± 6 b c	64.12 ± 4 b c
+ Clofibrate 200 µM	79.41 ± 4 b c	75.01 ± 5 b c	70.42 ± 4 b c
+ Clofibrate 400 µM	80.54 ± 4 b c	78.61 ± 5 b c	75.72 ± 5 b c

Hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer pH 7.4 at 37 °C.
ΔΨm was determined as the difference in rhodamine 123 uptake by control and test cells and expressed as fluorescence intensity unit.
Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey's HSD test.
FeIII:HQ I: (8-hydroxy- quinoline12.5µmol+Fe 1.5 µmol)
FeIII:HQ II: (8-hydroxy- quinoline 25µmol+Fe 3µmol)
[a]Significant compared with FeII:HQ I ($p < 0.05$).
[b]Significant compared with FeII:HQ II ($p < 0.05$).
[c]Significant compared with the same dose of the analogue ($p < 0.05$).

Mitochondrial membrane potential (Δψm)

Table 3 demonstrates the relative percentages of mitochondrial membrane potential (Δψm) in test groups and their control group, where the 100 percent of rhodamine 123 trapping occured in the intact mitochondria. Our results revealed that Fe III: 8HQ complexes caused a rapid decline of mitochondrial membrane potential as an apparent marker of mitochondrial dysfunction at 3 hours. Meanwhile, mitochondrial membrane potential decrease was prevented by pretreatment of hepatocytes with clofibrate and/or silafibrate, 20 minutes before adding cytotoxic complexes (FeIII:8HQ I, II). Further, there was a significant difference ($p < 0.05$) between equal concentrations of clofibrate and silafibrate in protection of mitochondrial membrane potential (Δψm).

Determination of reactive oxygen species "ROS"

The involvement of "ROS" in the cytotoxic mechanism was also studied that has been shown in Table 4. These data demonstrate that incubation of hepatocytes with Fe(III)⁄8-hydroxyquinoline complex at I, II concentration induced cytotoxicity proceeded ROS formation, and mitochondrial toxicity.

Clofibrate at concentrations of 100, 200, and 400 μM and silafibrate at concentrations of 100, 200, and 400 μM significantly ($p < 0.05$) prevented Fe III:8HQ complex I induced hepatocyte ROS formation. In addition, silafibrate markedly protects hepatocytes in comparison with equal doses of the parent analog, clofibrate.

Clofibrate at concentrations of 100 μM and silafibrate at concentrations of 100, 200, and 400 μM significantly ($p < 0.05$) prevented Fe III:8HQ complex II induced hepatocyte ROS formation. Meanwhile, silafibrate markedly caused more protection of hepatocytes in comparison with equal doses of the parent analog, clofibrate. Further, clofibrate did not have a protective effect in 200 and 400 μM concentrations against Fe III: 8HQ complex II induced ROS formation.

Table 4. Comparison of the effects of clofibrate and/or silafibrate on ROS generation in FeII:HQ complex-treated hepatocytes

Compounds added	DCF (%)		
	60 min	120 min	180 min
Control	100±5	111 ± 5	122 ± 4
+FeIII:HQ I	131 ± 5*	148 ± 6*	169 ± 5*
+ Silafibrate 100 μM	102 ± 7 a	115 ± 6 a	127 ± 6 a
+ Silafibrate 200 μM	54 ± 5 a c	79 ± 5 a c	96 ± 6 a c
+ Silafibrate 400 μM	96 ± 5 a c	103 ± 5 a c	114 ± 7 a c
+ Clofibrate 100 μM	112 ± 7 a c	128 ± 6 a c	143 ± 5 a c
+ Clofibrate 200 μM	123 ± 4 a c	134 ± 4 a c	151 ± 5 a c
+ Clofibrate 400 μM	110 ± 7 a c	131 ± 7 a c	154 ± 5 a c
+FeIII:HQ II	146 ± 5*	159 ± 5*	178 ± 5*
+ Silafibrate 100 μM	109 ± 6 b c	128 ± 6 b c	127 ± 5 b
+ Silafibrate 200 μM	86 ± 5 b c	98 ± 5 b c	115 ± 6 b
+ Silafibrate 400 μM	127 ± 5 b	138 ± 5 b	142 ± 6 b
+ Clofibrate 100 μM	119 ± 4 b c	135 ± 4 b c	148 ± 5 b c
+ Clofibrate 200 μM	131 ± 6 b	144 ± 6 b	166 ± 4 b
+ Clofibrate 400 μM	126 ± 5 b	142 ± 5 b	159 ± 5 b c

Hepatocytes were incubated with different compounds and ROS formation was measured at different time intervals. DCF formation was expressed as fluorescent intensity units (Shen et al., 1996).Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey's HSD test.
FeIII:HQ I: (8-hydroxy- quinoline12.5μmol+Fe 1.5 μmol)
FeIII:HQ II: (8-hydroxy- quinoline 25μmol+Fe 3μmol)
**Significant compared with control($p < 0.05$).*
[a]Significant compared with FeIII:HQI($p < 0.05$).
[b]Significant compared with FeIII:HQ II($p < 0.05$).
[c]Significant compared with the same dose of the analogue ($p < 0.05$).

DISCUSSION

In this study, we investigated the effect of a novel siliconized analog of clofibrate, silafibrate on isolated hepatocytes. It did not show significant toxicity in mentioned doses (Table 1). Therefore, we studied the hepatoprotective effects of clofibrate and its newly synthesized analog on the cytotoxicity of Fe (III) ⁄8-hydroxyquinoline complex. Earlier, Fe (III)⁄8-hydroxyquinoline was used to permeate and load hepatocytes with Fe (III) markedly increased cytotoxicity [28]. Iron and hydrogen peroxide are able to cause oxidation of a broad range

of substrates and induce biological damage. The reaction is known as the *Fenton reaction* and is complex and capable of producing both hydroxyl radicals and higher oxidation states of the iron [4].

It has been reported in various studies that the effects of fibrates on oxidative stress are extremely controversial. Some studies reported antioxidant activity of these drugs [7, 15, 26, 30, 39] , whereas others demonstrated no effect [5, 22, 33] or even prooxidant properties of fibrates [8, 24, 39].

Data obtained in the present study revealed that incubation of freshly isolated hepatocytes with clofibrate and its novel analog, silafibrate, demonstrate hepatoprotective effects against Fe III: 8HQ induced cytotoxicity.

It seems that PPARs agonists show antioxidant actions partly due to their effects on lipoprotein metabolism. They could elevate HDL levels that exert antioxidant and anti-inflammatory activities. PPAR-α activators increase Cu2+-Zn2+-superoxide dismutase and decrease p22 phox message expression in endothelial cells, suggesting that the drug may also exhibits antioxidant activity [14]. Moreover, other experiments revealed that fibrates may decrease the production of reactive oxygen species [27]. Other observations on isolated hepatocytes showed that direct addition of PPAR-α activators did not increase detectable ROS production [45].

In addition, previous studies showed that clofibrate could prevent acetaminophen (APAP) hepatotoxicity. Nicholls-Grzemski and colleagues demonstrated that hepatoprotection by clofibrate is not confined to APAP alone [10]. Activation of PPAR-α as a nuclear receptor has a key role in protection against liver injury of as structurally and mechanistically diverse hepatotoxicants as bromobenzene, chloroform, and carbon tetrachloride [19].

It seems that activation of PPAR-α by fibrates has a major role in their hepatoprotective effects against hepatotoxic chemicals. In the current study, we found that incubation of isolated hepatocytes with fibrates enhance their mitochondrial membrane potential and subsequently their viability. Beside this, we compared the effects of the novel analog, silafibrate, with its parent analog in freshly isolated hepatocytes. It revealed that silafibrate has better performance in hepatoprotective effects. A sila-replacement in clofibrate molecule altered the chemical and physicochemical properties. These changes modified bond lengths and angles, which altered the molecule's interaction with a receptor and, therefore, the pharmacological activity. Lipophilicity can be measured by log P, which reflects the equilibrium partitioning a molecule between an apolar (n-octanol) and a polar (water) phase [6]. We calculate log P value 3.32 for clofibrate and 5.14 for silafibrate with ACD/Labs package, release 7.0, Advanced Chemistry Development Inc Toronto Canada. An increase in lipophilicity should enhance permeability for the novel analog. This increase in lipophilicity of the novel analog may increase its pharmacological potency on activation of PPAR-α receptors.

It is likely that hepatoprotection is dependent on PPAR-α receptor activation. Previous experiments revealed that fibrates elevate the level of total antioxidants and lower MDA in blood [43]. These studies showed that activation of PPAR-*α* leads to an antioxidant effect by reducing plasma concentrations of malonyldialdehyde, a major sign of oxidative stress, and by stimulating the expression of SOD, one of the major molecules of antioxidant defense [43,46].

The mechanism of the antioxidant effect of fibrates is not yet clearly understood, but several possibilities can be considered. First, fibrates increased antioxidants and oxyradical scavenger enzymes protect against oxyradical-mediated cell death. Peroxisomes appear to have a ROS-mediated role in the oxidative reactions characteristic of senescence. The senescence-induced alterations in the ROS metabolism of peroxisomes are mainly characterized by the disappearance of catalase activity and an overproduction of $O_2^{\cdot-}$ and H_2O_2 and a strong reduction of APX and MDAR activities [21]. Second, PPAR-α modulate the expression of various proteins, by attenuate the selective arylation and/or by adjustment the effect of covalent binding on cellular integrity. Third, PPAR-α agonists may protect hepatocytes against external death protein that induced cell death in hepatocytes. Finally, PPAR-α activation induces the expression of cytochrome P450, which catalyzes some lipid peroxdation products, including 4-hydroxynonenal [9].

PPARs agonists promote mitochondrial proton gradient uncoupling, reduce ROS, and elevate heat generation, whereas they reduce lipotoxicity [1].

Mitochondrial membrane potential is a sensitive indicator of the activity of the mitochondrial proton pumps, electrogenic transport systems, and a key monitor for depolarization initiated cell death [23,31]. Clofibrate and silafibrate were not cytotoxic to intact rat hepatocytes, and there was no loss of cell viability over a 3 h incubation period (Table 1). Further, they offered strong protection against ROS formation in isolated hepatocytes (Table 4). On the other hand, silafibrate was more effective ($p < 0.05$) than clofibrate at inhibiting Fe(III)⁄8-hydroxyquinoline

induced hepatocyte membrane lyses after 3 h of incubation. These results demonstrate that the incubation of hepatocytes with PPARs agonists enhances the mitochondrial membrane potential. It seems that this reaction protects hepatocytes against cell death processes.

In conclusion, we found that the fibrates have a hepatoprotective effect against oxidative stress. Silafibrate has characteristic properties that demonstrated a better effect in hepatocytes protections. This might be because of its higher lipophilicity.

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