RESEARCH ARTICLE

Genotoxicity evaluation of HMG CoA reductase inhibitor rosuvastatin

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Abstract

The genotoxic potential of rosuvastatin as one of the statin drugs was assessed by chromosomal aberrations (CAs), micronucleus (MN) and DNA damage by comet assay in the human peripheral blood lymphocytes. Rosuvastatin was used at concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 μg/mL for these in vitro assays. In all assays, a negative and positive control were also included. CA frequencies were significantly increased in all concentrations at 24 hours and significantly increased in all concentrations except 0.0625 μg/mL at 48 hours, compared to the negative control. Rosuvastatin has a decreased mitotic index (MI) at 0.5- and 1-μg/mL concentrations at 24 hours and at 0.25, 0.5 and 1 μg/mL at 48 hours. A significant increase was observed for induction of MN in all treatments, compared to the negative control. Cytokinesis-block proliferation indices were not affected by treatments with rosuvastatin. In the comet assay, significant increases in comet tail length and tail moment were observed at 0.0625-, 0.5- and 1-μg/mL concentrations. Comet intensity was significantly increased in all concentrations except 0.0625 μg/mL. According to these results, rosuvastatin is cytotoxic and clastogenic/aneugenic in human peripheral lymphocytes. Further studies should be conducted in other test systems to evaluate the full genotoxic potential of rosuvastatin.

Keywords

Chromosomal aberrations, comet assay, genotoxicity, human blood lymphocytes, micronuclei, rosuvastatin, statin

Introduction

Statins are effective cholesterol-lowering agents that play a role by inhibiting the enzyme, 3-hydroxy 3-methylglutaryl coenzyme A (HMG CoA) reductase, which is the rate-controlling enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids. Statins have proved to be remarkably successful as therapeutic agents. The throughput from large-scale clinical trials has established the impressiveness of statin therapy in the primary and secondary protection of cardiovascular diseases (CVDs) associated with hypercholesterolemia (CTT, 2005, 2008; Gotto & Grundy, 1999; Schmidt et al., 2007; Stalker et al., 2001). Statins inhibit an enzyme that is essential not only for cholesterol biosynthesis, but also for the synthesis of several other nonsterol precursors essential for normal cellular function (MacDonald & Halleck, 2004). However, pleotropic and additional benefit shown with more intensive statin therapy (Cannon et al., 2004; LaRosa et al., 2005; Pedersen et al., 2005), there has been a trend toward using higher doses of statin. Further, cholesterol lowering is currently recommended for a wide range of people at cardiovascular risk, including those with average and below-average lipid levels (BRITISH, 2005; NCEP, 2001). This change is leading to increased statin use and the use of more-intensive regimens. Hence, the safety of this group of drugs is of considerable importance.

Rosuvastatin is also a class of medications in HMG-CoA reductase inhibitors. It is a synthetic lipid-lowering agent for oral administration. The chemical name for rosuvastatin calcium is bis[(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl (methylsulfonyl)amino] pyrimidin-5-yl](3R,5S)-3,5-dihydroxy-hept-6-enoic acid] calcium salt and it is sparingly soluble in water and methanol as well as slightly soluble in ethanol (Figure 1) (FDA, 2010).

Rosuvastatin produces its lipid-modifying effects in two ways. First, it increases the number of hepatic low-density lipoprotein (LDL) receptors on the cell surface to enhance uptake and catabolism of LDL. Second, rosuvastatin inhibits hepatic synthesis of very-low-density lipoprotein. Rosuvastatin has significantly greater efficacy in lowering LDL cholesterol and non-high-density lipoprotein cholesterol concentrations than other five statins. It has been shown to enable more patients to reach their LDL cholesterol goals than other statins and to do so with an acceptable safety profile (FDA, 2010; McKenney, 2005; McTaggart et al., 2001).

In the scientific literature review that we made, we have not found any genotoxicity studies about rosuvastatin except for U.S. Food and Drug Administration (FDA) reports. In these reports, nonclinical toxicological throughputs about rosuvastatin were presented. In these reports, the incidence
of uterine stromal polyps in rats exposed to 2, 20, 60 or 80 mg/kg/day of rosuvastatin by oral gavage was significantly increased in females at 80 mg/kg/day. Increased incidence of polyps was not observed at lower doses (FDA, 2010). In a carcinogenicity study in mice given 10, 60 and 200 mg/kg/day by oral gavage, an increased incidence of hepatocellular adenoma (HCA) and carcinoma (HCC) was observed at 200 mg/kg/day. This mice carcinogenicity study is in agreement with rat carcinogenicity studies, and increased incidence of hepatocellular tumors was not observed at lower doses. The FDA also suggested that rosuvastatin was not mutagenic or clastogenic with or without metabolic activation in the Ames test with Salmonella typhimurium and Escherichia coli, the mouse lymphoma assay and the chromosomal aberration (CA) assay in Chinese hamster lung (CHL) cells. Rosuvastatin was negative in the in vivo mouse micronucleus (MN) test (FDA, 2010).

There are many test methods used to detect the potential genotoxicity of different chemicals. CAs, both in vivo and in vitro, and single-cell gel electrophoresis (SCGE) are very sensitive methods (Yilmaz et al., 2008). Hence, in the present study, we planned to determine the genotoxic potential of rosuvastatin in vitro in human lymphocyte cultures to increase knowledge on the genotoxic activity of this cholesterol drug with CAs, MN and DNA damage by comet assay.

Methods

Chemicals

Rosuvastatin was isolated from one of the commercial statin drugs. For this purpose, first, drugs were treated with 100 mL of methanol and put on a magnetic stirrer for 20 hours. Then, the solution was filtered with filter paper and was evaporated by Heidolph 4000. Purity of rosuvastatin was determined by 1H NMR (nuclear magnetic resonance) spectrum and melting point. The results show that the purity of rosuvastatin is >98%. The 1H NMR data and melting point (155–165 °C) of rosuvastatin corresponded to those in the literature (Dent, 2007; Fischer et al., 2010).

CA analysis in human lymphocytes

Heparinized peripheral blood samples of 4 healthy nonsmoking (2 male and 2 female) donors were cultured in chromosome medium B and treated with 0.0625-, 0.125-, 0.25-, 0.5- and 1-µg/mL concentrations of rosuvastatin. An untreated and a positive control mytomycin C (MMC; 0.2 µg/mL) were also maintained in all experiments. Cells in culture were exposed to test substance for 24 and 48 hours. Cultures were incubated for 72 hours at 37 °C, and colchicine (final concentration: 0.06 µg/mL) was added to each culture 2 hours before harvesting. Cells were then harvested by centrifugation (1200 rpm for 10 minutes), and the pellet was treated with 0.075 M of KCl for 30 minutes at 37 °C. Cells were centrifuged again and fixed in cold methanol/acetic acid (3:1). The fixation process was repeated three times. Slides were stained with 5% Giemsa (pH = 6.8) in Sorensen buffer for 20–25 minutes, washed in distilled water, dried at room temperature and mounted with Depex.

MN test in cultured human lymphocytes

Whole blood was added to 2.5 mL of chromosome medium B (Biochrom AG, Berlin, Germany). Human lymphocytes were incubated at 37 °C for 72 hours and treated with rosuvastatin at 0.0625, 0.125, 0.25, 0.5 and 1 µg/mL for 48 hours. Cytoscalasin B (5.2 µg/mL) was added to arrest cytokinesis at 44 hours after the start of culture. Then, cells were harvested by centrifugation (1000 rpm for 10 minutes), and the pellet was treated with hypotonic solution (0.075 M of KCl) for 5 minutes at 4 °C. Cells were recenterfuged and fixed three times in cold methanol/acetic acid (3:1). In the last fixative, 1% formaldehyde was added to preserve the cytoplasm. Slides were prepared by dropping and air-drying. Slides were stained with 5% Giemsa (pH = 6.8) in Sorensen buffer for 13–15 minutes, washed in distilled water, dried at room temperature and mounted with Depex.

Comet assay in cultured human lymphocytes

The comet assay (SCGE) was conducted under alkaline conditions according to Singh et al. (1988). Heparinized peripheral blood was obtained before the performance of the test. Lymphocytes were isolated by Biocoll separating solution. To detect the viability of cells, the trypan blue exclusion test was used. Cell viability was >99%. Isolated human lymphocytes were incubated with 0.0625-, 0.125-, 0.25-, 0.5- and 1-µg/mL concentrations of rosuvastatin for 1 hour at 37 °C. Negative and positive controls (H2O2; 3.4 µg/mL) were also included at the same temperature and exposure time in parallel with rosuvastatin. The other procedure was applied according to Mamur et al. (2010).

Slide evaluation

In human lymphocytes, 100 well-spread metaphases per donor (total, 400 metaphases per concentration) were analyzed. The mitotic index (MI) was also determined by scoring 3000 cells from each donor (total, 12,000 cells per concentration). Micronuclei were scored from 1500 binucleated cells per donor (total, 6000 binucleated cells per concentration). Cell proliferation was evaluated using the cytokinesis-block proliferation index (CBPI), which indicates the average number of cell cycles. Five hundred lymphocytes (total, 2000 lymphocytes per concentration) were scored to evaluate the percentage of cells with one, two, three and four nuclei. The CBPI was calculated.
according to Surrales et al. (1995) as follows: \( [1 \times N1] + [2 \times N2] + [3 \times (N3 + N4)]/N \), where \( N1-N4 \) represent the number of cells with one to four nuclei, respectively, and \( N \) is the total number of cells scored. In the comet assay, slides were examined using a fluorescent microscope (BAB research microscope; BAB Imaging System, Ankara, Turkey). Slides were prepared for each concentration of rosuvastatin. The comet parameters used for the evaluation are tail length, tail DNA\% and tail moment. For this reason, 100 comets on each slide were analyzed with a specialized image analyses system (BS 200 ProP; BAB Imaging System) to determine these parameters.

**Statistical analysis**

For the statistical analysis of the results, \( z \)-test for percentage of abnormal cell, CA/cell, CBPI, MI, MN was used. \( t \)-test was performed to determine the possible DNA damage induced by rosuvastatin. Concentration-response relationships were determined to find out the possible DNA damage induced by rosuvastatin. For the statistical analysis of the results, \( z \)-test for percentage of abnormal cell, CA/cell, CBPI, MI, MN was used. For the statistical analysis of the results, \( z \)-test for percentage of abnormal cell, CA/cell, CBPI, MI, MN was used. For the statistical analysis of the results, \( z \)-test for percentage of abnormal cell, CA/cell, CBPI, MI, MN was used.

### Results

To evaluate the effect of HMG-CoA reductase inhibitor rosuvastatin on human lymphocytes, three parameters (CAs, MN and comet) were analyzed. The results reported in Table 1 show the induction of CAs after treatment with increasing concentrations of rosuvastatin. Rosuvastatin induced six types of CAs: chromatid and chromosome breaks; fragments; sister-chromatid union; dicentric chromosomes and chromatid exchange. Chromatid breaks were observed as the most common aberrations, whereas chromosome breaks and fragments were also observed respectively. Sister-chromatid union and dicentric chromosome abnormalities were only observed in the 24-hour treatment period. The CA/cell ratio significantly increased in all concentrations in two treatments except 0.0625 µg/mL at 48 hours, when compared to the controls.

Rosuvastatin decreased the CBPI, but these results showed that the effect was statistically insignificant. Rosuvastatin reduced the percentage of MI significantly at 0.5- and 1-µg/mL concentrations at 24 hours. In addition to this, it

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Period (hours)</th>
<th>Doses (µg/mL)</th>
<th>Abnormal cell ± SE (%)</th>
<th>CAs/cell ± SE</th>
<th>MI ± SE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>0.00</td>
<td>1</td>
<td>0.25 ± 0.25</td>
<td>0.002 ± 0.003</td>
</tr>
<tr>
<td>Positive control</td>
<td>24</td>
<td>0.20</td>
<td>118</td>
<td>36.50 ± 2.41</td>
<td>0.470 ± 0.025</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>24</td>
<td>0.0625</td>
<td>9</td>
<td>3.75 ± 0.95</td>
<td>0.038 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>23</td>
<td>3</td>
<td>6.50 ± 1.23c</td>
<td>0.065 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>17</td>
<td>2</td>
<td>5.75 ± 1.16c</td>
<td>0.058 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>32</td>
<td>8</td>
<td>10.25 ± 1.52c</td>
<td>0.105 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>18</td>
<td>5</td>
<td>7.25 ± 1.30c</td>
<td>0.073 ± 0.013</td>
</tr>
<tr>
<td>Control</td>
<td>48</td>
<td>0.00</td>
<td>8</td>
<td>2.00 ± 0.70</td>
<td>0.020 ± 0.007</td>
</tr>
<tr>
<td>Positive control</td>
<td>48</td>
<td>0.20</td>
<td>529</td>
<td>75.25 ± 2.16</td>
<td>1.878 ± 0.680</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>48</td>
<td>0.0625</td>
<td>12</td>
<td>3.25 ± 0.89</td>
<td>0.033 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>19</td>
<td>–</td>
<td>4.50 ± 1.04a</td>
<td>0.048 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>24</td>
<td>2</td>
<td>7.00 ± 1.28c</td>
<td>0.073 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>49</td>
<td>4</td>
<td>11.75 ± 1.61c</td>
<td>0.135 ± 0.017</td>
</tr>
</tbody>
</table>

Four hundred metaphases were scored for each treatment for CAs, and 12,000 metaphases were scored for each dose level for the MI. 

\( a \)Significantly different from the negative control; \( p < 0.05 \) \( z \)-test. \( b \)Significantly different from the negative control; \( p < 0.01 \) \( z \)-test. 

\( c \)Significantly different from the negative control; \( p < 0.001 \) \( z \)-test.

Table 1. Total chromosomal aberrations in human lymphocytes treated with rosuvastatin.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Period (hours)</th>
<th>Doses (µg/mL)</th>
<th>MN/cell (%) ± SE</th>
<th>CBPI ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>48</td>
<td>0.00</td>
<td>0.12 ± 0.05</td>
<td>1.41 ± 0.26</td>
</tr>
<tr>
<td>Positive control</td>
<td>48</td>
<td>0.20</td>
<td>17.47 ± 0.49</td>
<td>1.19 ± 0.24</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>48</td>
<td>0.0625</td>
<td>0.42 ± 0.08c</td>
<td>1.26 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>25</td>
<td>0.85 ± 0.12d</td>
<td>1.34 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>37</td>
<td>0.95 ± 0.13d</td>
<td>1.31 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>32</td>
<td>1.03 ± 0.13d</td>
<td>1.22 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>72</td>
<td>1.27 ± 0.15d</td>
<td>1.13 ± 0.24</td>
</tr>
</tbody>
</table>

\( a \)Cell with 14 micronuclei. 

\( b \)Cell with six micronuclei.

\( c \)Significantly different from the control; \( p < 0.01 \) \( z \)-test. 

\( d \)Significantly different from the control; \( p < 0.001 \) \( z \)-test.

BN, binucleated; SE, standard error.
also reduced MI at all concentrations except 0.0625 μg/mL at 48 hours, compared to negative control.

To evaluate possible clastogenic and/or aneugenic effects, the cytokinesis-block MN assay was conducted. Rosuvastatin increased the frequency of lymphocytes with micronuclei. Micronuclei frequency significantly increased in all concentrations (Table 2).

The rise in MN frequency nearly reached 11-fold, compared to the negative control, at 1 μg/mL. Most cells showed just one micronucleus, seven cells with two micronuclei, one cell with three micronuclei, one cell with six micronuclei (Figure 2) and one cell with 14 micronuclei (Figure 3). However, in this assay, we observed an apoptotic cell.

In the comet assay, tail length and tail moment showed significant increases at 0.0625-, 0.5- and 1-μg/mL concentrations, when we applied rosuvastatin to isolated human lymphocytes. These increases were dose dependent (both $r = 0.52$), and in tail intensity, a significant increase was observed at all concentrations except 0.0625 μg/mL. This increase was also dose dependent ($r = 0.81$; Table 3).

### Discussion

Rosuvastatin has joined other statins that are used in lowering LDL cholesterol, treating dyslipidemia and reducing the risk of coronary heart disease (CHD). The importance of cholesterol lowering in CHD risk reduction has been established in numerous randomized, clinical trials (SSSSG, 1994; Tonkin et al., 1998). Statins may also avail in the therapy of other disorders, including Alzheimer’s disease, osteoporosis and, especially, cancer (Chan et al., 2003; Crisby et al., 2002; Waldman & Kritharides, 2003). Ertugrul et al. (2011) demonstrated that 25-hydroxyvitamin D level (a protector against CVD) was increased with rosuvastatin treatment.

Verreth et al. (2007) pointed out that in vitro administration of rosuvastatin restored superoxide dismutase 1 expression in THP-1 macrophages and foam cells. The latter was associated with less-oxidized LDL accumulation within atherosclerotic plaques and inhibition of plaque progression.

Rosuvastatin is not only a lipid lowering drug. It could significantly decrease oxidative stress (OS) and has immunomodulatory properties in a dose- and LDL-independent manner (Resch et al., 2006). Ajith et al. (2008) suggest that rosuvastatin (1.5 or 2 mg/mL) is able to protect against lipid peroxidation (LPO). Further, the investigators reported that H$_2$O$_2$-induced changes in pBR322 plasmid DNA and fragmentation of hepatic DNA were alleviated by rosuvastatin. However, rosuvastatin did not show any superoxide anion-scavenging activity. Kamigaki et al. (2011) suggest that statins may be a new cholangiocarcinoma treatment option that could potentially enhance the anticancer effect of preexisting anticancer drugs. These studies show that rosuvastatin has many beneficial pleotropic effects. Compared to our study, lowering of OS and LPO, reducing H$_2$O$_2$-induced DNA fragmentation and anticancer effect by rosuvastatin are not in agreement with our results. In our study, rosuvastatin has genotoxic potential in in vitro human lymphocytes.

### Table 3. Assessment of DNA damage by comet assay after in vitro exposure of human lymphocytes to rosuvastatin.

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Period (hours)</th>
<th>Doses (μg/mL)</th>
<th>Tail length (μm)</th>
<th>Tail moment</th>
<th>Tail intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>0</td>
<td>8.012 ± 0.45</td>
<td>6.262 ± 0.44</td>
<td>234.771 ± 0.23</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>1</td>
<td>0.0625</td>
<td>9.923 ± 0.70*</td>
<td>8.203 ± 0.68*</td>
<td>234.602 ± 0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.125</td>
<td>9.446 ± 0.57</td>
<td>7.520 ± 0.55</td>
<td>236.375 ± 0.23*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.250</td>
<td>7.222 ± 0.46</td>
<td>5.488 ± 0.44</td>
<td>236.096 ± 0.18*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>11.430 ± 0.83*</td>
<td>9.732 ± 0.82*</td>
<td>236.113 ± 0.26*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>10.438 ± 0.69*</td>
<td>8.628 ± 0.68*</td>
<td>237.187 ± 0.25*</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>1</td>
<td>3.4</td>
<td>61.931 ± 2.13</td>
<td>59.599 ± 2.13</td>
<td>244.239 ± 0.23</td>
</tr>
</tbody>
</table>

*aSignificantly different from the negative control; $p < 0.05$ ($t$-test).
Genotoxicity evaluation of rosuvastatin

The comet assay detects strand breaks. Generation of DNA damage is considered to be an important initial event in carcinogenesis (Møller, 2005). The alkaline version of the method is widespread and frequently used for detecting genetic damage induced by different genotoxic agents, such as radiation, pesticides and chemical compounds.

Chromosomal breaks or interference with the mitotic process, resulting in lagging of the chromosomal material during cell division, leads to the MN (Gudi et al., 1990). In this study, to evaluate possible clastogenic and/or aneugenic effects, the cytokinesis-block MN assay was conducted. In our results, rosuvastatin significantly increased micronuclei in all concentrations. After these results, we suggested that rosuvastatin could have resulted in clastogenic and/or aneugenic effects in vitro. On the other hand, in this study, we did not observe any polyploidy in CA analyses. For this reason, to understand whether rosuvastatin has an aneugenic effect, the MN/fluorescence in situ hybridization assay should be conducted. However, in the present study, we did not observe any nucleoplasmic bridges and nuclear buds.

Link et al. (2011) showed that rosuvastatin treatment in patients with acute coronary syndrome resulted in the expression of B-cell lymphoma 2 (antiapoptotic protein) being down-regulated within 24–72 hours in CD4+ T cells. The number of apoptotic CD4+ CD28null T cells remained low on placebo, but on statins, these apoptotic T cells increased. Compared to placebo, this difference was statically significant. Already, after 72 hours, CD4+ CD28null T cells of patients treated with rosuvastatin entered an apoptotic pathway. Likewise, in our study, with administration of rosuvastatin, we detected apoptotic cells on human lymphocytes with the MN assay.

Conclusions

Rosuvastatin induces cytotoxicity, clastogenicity and DNA effects in human lymphocytes in vitro. However, it should be investigated in other mammalian test system(s) for its genotoxic effects. Further, biomonitoring studies should also be conducted with patients receiving therapy with this drug.

Declaration of interest

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References


