IJPSR (2021), Volume 12, Issue 2 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 25 February 2020; received in revised form, 13 May 2020; accepted, 22 May 2020; published 01 February 2021

IN-VITRO EVALUATION OF PLEIOTROPIC PROPERTIES OF ROSUVASTATIN

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Keywords:

Rosuvastatin, *In-vitro*, Anti-oxidant, Anti-inflammatory, Anti-microbial

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ABSTRACT: Rosuvastatin, a selective and competitive inhibitor of enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, is marked as a first-choice drug amongst lipid-lowering agents because of its unique pharmacological and pharmacokinetic properties. An additional advantage of rosuvastatin administration is its specific, non-lipid pleiotropic properties, which are considered promising new fields of research for potential new therapeutic implications of this class of drugs. The aim of this study was to evaluate the antioxidant, anti-inflammatory, and anti-microbial activity of rosuvastatin with the application of the most commonly used *in-vitro* assays. Rosuvastatin was dissolved in dimethyl sulfoxide and prepared as a range of different concentrations. The activity of rosuvastatin was compared to well-known antioxidant, anti-inflammatory, and antimicrobial agents. For determination of antioxidant activity, a spectro-photometric DPPH radical scavenging assay was used, while inhibition of heat-induced protein denaturation was used for evaluation of anti-inflammatory activity of tested rosuvastatin solutions. Evaluation of antimicrobial activity was performed with the application of agar-well diffusion assay against both Gram-positive and Gram-negative bacteria as well as the fungi. The results of this *in-vitro* study suggest that tested rosuvastatin solutions at various concentrations demonstrate antioxidant, anti-inflammatory, and antimicrobial properties. Present findings indicate that the expression of pleiotropic properties of rosuvastatin is dependent upon the concentration of rosuvastatin. However, it should be noted that findings from this study are restricted to *in-vitro* assays, and there is clearly a need for carrying further research involving *in-vivo* models.

INTRODUCTION: Rosuvastatin, the latest member of the statin class, is a selective and competitive inhibitor of the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase).

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(2).1201-06

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(2).1201-06

With its unique pharmacological and pharmaco-kinetic properties, rosuvastatin shows the properties of an "ideal statin," which implies that in low doses, it effectively lowers lipid levels, exhibits the maximum effect of inhibiting HMG-CoA reductase at the target site in hepatocytes, possess a longer elimination half-life, lower systemic availability and rarely interacts with other drugs [1].

Different clinical trials have shown that administration of statins results in a more effective reduction of cardiovascular risk than other non-statin hypolipidemics.

That is explained by a series of specific, non-lipid pleiotropic properties, which brings an additional advantage to statins compared to other hypo-lipidemics [2-6]. The mechanism of pleiotropic activity is primarily based on the prevalence of the enzyme HMG-CoA reductase in almost every cell of the body.

Therefore, statins, besides to inhibiting the action of HMG-CoA reductase in hepatocytes, also blocks the enzyme in endothelial cells, myocyte, and inflammatory cells. However, the reduction of isoprenoid synthesis in the process of cholesterol biosynthesis in hepatocytes due to the inhibition of HMG-CoA reductase is considered to be the most significant driver of statin pleiotropic activity [7].

Pleiotropic properties of statins are promising new fields of research for a potential new therapeutic implication of this class of drugs. In recent years, various studies were published underlining the anti-inflammatory and antioxidant activity of the statins [6, 7, 8]. Furthermore, it has been found that statins interfere with oxidation in several pathways that result in the reducing process of atherosclerosis [9].

Also, it has been confirmed that statins inhibit different inflammation markers such as C-reactive protein [10], interleukins, interferons, and tumor necrosis factor-α [11, 12].

Different *in-vivo* and human studies have shown that individuals treated with statins are less prone to bacterial infections underlining its antimicrobial activity as well [13, 14, 15]. This study aimed to evaluate the antioxidant, anti-inflammatory, and antimicrobial activity of rosuvastatin with an application of the most commonly used *in-vitro* assays.

MATERIALS AND METHODS: Rosuvastatin calcium standard (99.1% purity; Lot No: BM14000510) was obtained from ILKO ILAC SANAYII VE TICARET A.S. Ethanol (puriss. p.a., ≥99.8%) and 1, 1-diphenyl-2-picriyhydrayil radical (DPPH) were purchased from Sigma-Aldrich; nutrient agars and antibiotic discs were purchased from Laboratories Conda S.A., and dimethyl sulfoxide (DMSO) from Semikem, BiH. All other chemicals used were of the highest analytical grade available. Spectrometer Lambda 25 UV/VIS, PerkinElmer was used for measuring absorbance and incubator Lab-Line Imperial III, Barnstead, USA for incubation of Petri plates.

***In-vitro* Antioxidant Activity:** Antioxidant activity was evaluated with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Rosuvastatin was dissolved in DMSO, and tested solutions were prepared in the following concentrations: 0.10, 0.25, 0.50, 0.75, and 1.00 mg/ml. Ascorbic acid was used as a standard and prepared at the same concentrations as the tested solutions of rosuvastatin. The reaction mixture consisted of 2.5 ml of tested solution and 1.0 ml of an ethanolic solution of DPPH in concentration 3×10^{-4} mol/L.

The test tubes were stored in a dark place and kept at room temperature for 20 min, after which the absorbance was measured at 517 nm. The absorbance of the ethanolic solution of DPPH was also measured and served as a control solution. An experiment was performed in triplicate. Antioxidant activity was expressed as Radical Scavenging Capacity (RSC) and calculated using the following formula:

$$RSC\ (\%) = 100 - (A_s \times 100/A_c)$$

Where A_s is the absorbance of the sample and A_c is the absorbance of the control solution. Based on the graphically presented equation curve, in dependence on the concentration of the tested solutions (mg/ml) and RSC (%), results were presented as IC_{50} values.

***In-vitro* Anti-inflammatory Activity:** Inhibition of heat-induced protein (egg albumin) denaturation was used for evaluation of the anti-inflammatory activity of rosuvastatin tested solutions. Rosuvastatin was dissolved in DMSO, and tested solutions were prepared in the following concentrations: 0.10, 0.25, 0.50, 0.75, and 1.00 mg/ml. Acetylsalicylic acid was used as a standard and prepared at the same concentrations as the tested solutions of rosuvastatin.

The reaction mixture consisted of 2.0 ml of tested solution, 2.8 ml phosphate buffer saline with adjusted pH 6.4, and 0.2 ml of egg albumin. The same reaction mixture served as the control, except the tested solution was replaced with distilled water. Mixtures were incubated for 15 min at 37 °C ± 2 °C and then heated for 5 min at 70 °C. After

cooling down at room temperature, their absorbance was measured at 660 nm [16].

An experiment was performed in triplicate. Anti-inflammatory activity was expressed as the inhibition of protein denaturation (%) and calculated by using the following formula:

$$\text{Inhibition (\%)} = 100 \times (1 - A_s/A_c)$$

Where A_s is the absorbance of samples and A_c is the absorbance of control.

***In-vitro* Antimicrobial Activity:** Agar well diffusion assay was used for evaluation of the antibacterial activity of both Gram-positive and Gram-negative bacteria, as well as the antifungal activity. The following strains were obtained from the American Type of Culture Collection (ATCC): *Staphylococcus aureus* (ATCC6538), *Listeria monocytogenes* (ATCC 35152), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC8739), and *Candida albicans* (ATCC10231). For preparing inoculums, suspensions of microorganisms were adjusted on 0.5 McFarland standards (approximately 1.5×10^8 CFU/ml) and cultured on nutrient agar. With the usage of sterile borer, wells with 6 mm diameter were created on agar in every Petri plate. The solution of rosuvastatin was prepared by dissolving in DMSO at the following concentrations: 0.10, 0.25, 0.35, and 0.50 mg/ml.

A volume of 100 µl of tested solutions was added into wells, as well as the DMSO, which served as a negative control. The following standard antibiotics served as positive control: Chloramphenicol (30 µg/disc), Ceftizoxime (30 µg/disc), Amikacin (30 µg/disc), and Nystatin (100 µg/disc). Then, Petri plates with bacteria were incubated at 37 °C for 18-24 h and plates with fungi at 25 ºC for 48 h. After the incubation period, the diameter of the resulting zone of inhibition was measured. An experiment was performed in triplicate, and obtained values of the diameter of zone of inhibition were proportional to the degree of antimicrobial sensitivity of tested solutions.

RESULTS AND DISCUSSION:

***In-vitro* Antioxidant Activity:** Antioxidant activity is defined as the ability of a molecule to protect a cell from oxidation or counteract harmful effects of free radicals caused by oxidative stress [17]. Various *in-vitro* assays have been developed to determine the antioxidant activity of pharmacologically active substances [18]. DPPH radical scavenging assay is fast, cheap, and one of the most commonly used *in-vitro* assays for evaluation of antioxidant activity. This assay is based on the reaction between molecule or antioxidant and stable free radical DPPH, which results in the discoloration of molecule [19]. In our study, DPPH radical scavenging assay was used for the determination of antioxidant activity of rosuvastatin and ascorbic acid at a different range of concentrations. Both rosuvastatin and ascorbic acid demonstrated maximum radical scavenging of 46.73% and 68.51% at a concentration of 1.0 mg/ml, respectively **Fig. 1**.

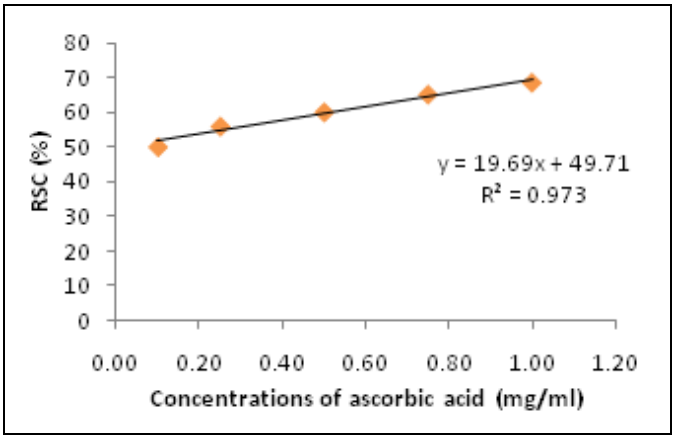

FIG. 1: DPPH RADICAL SCAVENGING ASSAY OF ASCORBIC ACID AND ROSUVASTATIN

Compared to test solutions of ascorbic acid, all tested solutions of rosuvastatin showed less antioxidant activity. Furthermore, results from our study showed that the percentage of RSC increases with an increase in rosuvastatin concentrations. Therefore, our findings imply that antioxidant effects of rosuvastatin are dose dependant. The IC_{50}

values represent the concentration of the tested solution required to inhibit/reduce the initial DPPH radical concentration by 50%.

The IC_{50} was calculated from the concentration-response curve. For ascorbic acid, IC_{50} was found to be 0.04 mg/ml, and for rosuvastatin, IC_{50} was found to be 1.45 mg/ml. As expected, lower IC_{50} values of ascorbic acid indicate higher antioxidant activity.

***In-vitro* Anti-inflammatory Activity:** The process of protein denaturation is well-known and described cause of inflammation. In this study, we investigated the ability of different concentrations of rosuvastatin solutions to inhibit protein denaturation. Anti-inflammatory activity was determined with the application of *in-vitro* spectro-photometric assay of heat-induced denaturation of egg albumin. This is a commonly used *in-vitro* assay, which is considered a convenient screening method for potential anti-inflammatory drugs [20].

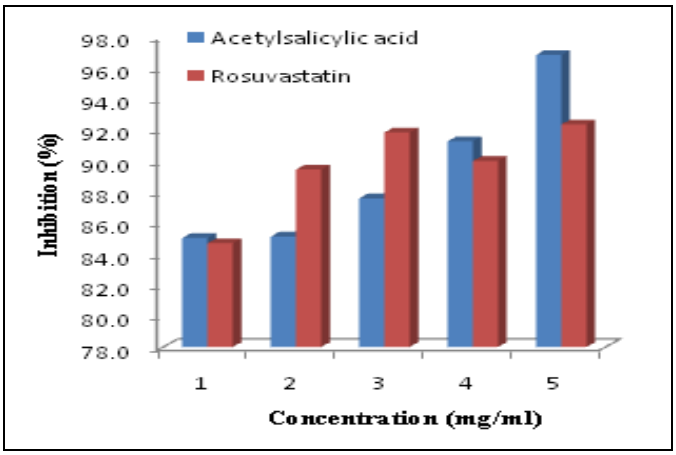

FIG. 2: INHIBITION OF EGG ALBUMIN DENATURATION OF ACETYLSALICYLIC ACID AND ROSUVASTATIN

Results from the study revealed that tested solutions of rosuvastatin showed mean inhibition of egg albumin denaturation of 92.36, 90.00, 91.84, 89.44, and 84.69% for concentrations of 1.00, 0.75, 0.50, 0.25, and 0.10 mg/ml, respectively. Acetyl-salicylic acid, which was used as control, showed inhibition of egg albumin denaturation of 96.84, 91.27, 87.59, 85.12 and 85.03% for concentrations of 1.00, 0.75, 0.50, 0.25 and 0.10 mg/ml, respectively **Fig. 2**. Both solutions of rosuvastatin and acetylsalicylic acid demonstrated maximum protein inhibition of 92.36% and 96.84% at the concentration of 1.00 mg/ml. Based on the obtained results, it is interesting to note that lower concentrations of rosuvastatin solution (0.25 and 0.50 mg/ml) showed a higher percentage of protein denaturation when compared to equal concentrations of acetylsalicylic acid solutions. Findings from our study indicate a positive dose dependant response for rosuvastatin solutions at lower concentrations. Due to the fact that tested solutions of rosuvastatin showed a similar percentage of inhibition of protein denaturation compared to the solution of acetylsalicylic acid, it can be stated that rosuvastatin is as efficient anti-inflammatory agent as an acetylsalicylic acid in tested concentrations.

***In-vitro* Antimicrobial Activity:** In reducing the global spread of infectious diseases and antibiotic resistance, detection of potential sources of novel antimicrobial agents is of crucial importance. As the current antimicrobials are simply not sufficient, further studies are based on the screening of existing drugs, with potential antimicrobial properties that could be used in supportive or combined anti-microbial therapy [21]. In this study, agar well diffusion assay was used to evaluate the potential antibacterial and antifungal activity of rosuvastatin. The antibacterial activity of rosuvastatin was tested against two strains of Gram-positive and Gram-negative bacteria, while antifungal activity was tested against *Candida albicans*. Measured diameters of zones of inhibition (mm) for tested antimicrobial activity of rosuvastatin solutions are given in **Table 1**.

The highest sensitivity to rosuvastatin was observed at a concentration of 0.50 mg/ml by *Staphylococcus aureus*, *Escherichia coli,* and *Candida albicans* resulting in a maximum zone of inhibition of 10.3, 10.0, and 9.7 mm, respectively. Results from our study indicate that *Pseudomonas aeruginosa* and *Listeria monocytogenes* are non-sensitive to all tested concentrations of rosuvastatin solution as no zones of inhibition were detected. As expected, DMSO (negative control) showed no antimicrobial activity against tested micro-organisms while antibiotics (positive control) demonstrated the most efficient inhibition of microbial growth **Table 2**. Compared to the positive control, antimicrobial activity of tested rosuvastatin solutions was less efficient in suppressing microbial growth. Furthermore, it was

noted that higher concentrations of rosuvastatin solutions resulted in a larger diameter of zones of inhibitions implying that antimicrobial activity of rosuvastatin is dependent upon the concentration.

Recent *in-vitro* novel antibacterial study by Al-Kuraishy *et al.,* confirmed significant antibacterial effects of rosuvastatin as well as significant additive effects of rosuvastatin with cefixime [22].

TABLE 1: ANTIMICROBIAL ACTIVITY OF TESTED ROSUVASTATIN SOLUTIONS

Tested rosuvastatin solutions (mg/ml)	Diameter of zone of inhibition (mm)				
	Escherichia coli **(ATCC8739)**	***Pseudomonas aeruginosa*** **(ATCC 9027)**	***Staphylococcus aureus*** **(ATCC6538)**	***Listeria monocytogenes*** **(ATCC 35152)**	***Candida Albicans*** **(ATCC 10231)**
0.10	8.7	ND	8.3	ND	8.0
0.25	9.3	ND	8.3	ND	8.3
0.35	9.3	ND	10.0	ND	8.7
0.50	10.0	ND	10.3	ND	9.7

*ND= not detected

TABLE 2: ANTIMICROBIAL ACTIVITY OF TESTED CONTROLS

Tested controls	Diameter of zone of inhibition (mm)				
	Escherichia coli **(ATCC8739)**	***Pseudomonas aeruginosa*** **(ATCC 9027)**	***Staphylococcus aureus*** **(ATCC6538)**	***Listeria monocytogenes*** **(ATCC 35152)**	***Candida Albicans*** **(ATCC 10231)**
CHL (30 µg/disc)	14.7	8.0	10.0	18.0	NT
ZOX (30 µg/disc)	10.3	ND	10.3	11.0	NT
AMK (3 0µg/disc)	10.3	18.3	20.0	18.7	NT
Nystatin (100 µg/disc)	NT	NDNT	NT	NT	15.7
DMSO negative control	ND	ND	ND	ND	NT

*CHL = Chloramphenicol, ZOX = Ceftizoxime, AMK = Amikacin, DMSO = dymethylsulfoxide, ND = not detected, NT = not tested

CONCLUSION: Findings from the present *in-vitro* study suggest that tested solutions of rosuvastatin at various concentrations demonstrate antioxidant, anti-inflammatory, and antimicrobial activity. Compared to well-known antioxidant, anti-inflammatory, and antimicrobial agents, solutions of rosuvastatin showed similar efficiency. Furthermore, a study showed that different concentrations of rosuvastatin solutions resulted in different expressions of pleiotropic properties. With the increase of rosuvastatin concentration, its pleiotropic activity increased and therefore implying on concentration-dependent response.

In conclusion, tested solutions of rosuvastatin have demonstrated significant *in-vitro* pleiotropic activity confirming that administration of this drug can go beyond the treatment of hyperlipidemia. However, it should be noted that findings from this study are restricted to *in-vitro* assays, and there is clearly a need for carrying further research involving *in-vivo* models.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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How to cite this article:
Pehlivanović B, Čaklovica K, Lagumdžija D, Smajić NŽ and Bečić F: *In-vitro* evaluation of pleiotropic properties of rosuvastatin. Int J Pharm Sci & Res 2021; 12(2): 1201-06. doi: 10.13040/IJPSR.0975-8232.12(2).1201-06.