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Research Article
Stimulation of 5-Hydroxytriptamine 7 Receptor by LP-211 Boosts Angiogenic Response

[1]Fatih Yesildal, [2]Suat Doganci, [3]Vedat Yildirim and [4]Taner Ozgurtas

[1]Department of Medical Biochemistry, Istanbul Medeniyet University, Goztepe Education and Research Hospital, Kadikoy, 34722 Istanbul, Turkey
[2]Department of Cardiovascular Surgery, University of Health Sciences, Gulhane Education and Research Hospital, Ankara, Turkey
[3]Department of Anesthesiology, University of Health Sciences, Gulhane Education and Research Hospital, Ankara, Turkey
[4]Department of Medical Biochemistry, University of Health Sciences, Gulhane Education and Research Hospital, Ankara, Turkey

Abstract

Background and Objective: The 5-hydroxytriptamine type 7 receptors (5-HT7Rs) belong to the serotonin receptor family and modulate many processes in organism and are also found in membrane of endothelial cells (ECs). The LP-211 is a selective agonist of 5-HT7Rs. The purpose of this study was to investigate the effects of activated 5-HT7Rs by LP-211 on regulation of angiogenesis. **Materials and Methods:** The effect of LP-211 was tested *in vitro* by cell proliferation and matrigel assays and *in vivo* by chick chorioallantoic membrane (CAM) assay. **Results:** Cell proliferation assay showed that LP-211 had a positive effect on EC proliferation. The LP-211 activated migration of ECs and promoted angiogenesis in matrigel assay *in vitro*. Stimulation of 5-HT7R by LP-211 caused a significant increase in angiogenic response compared to the control group in CAM assay *in vivo*. The LP-211, selective agonist of 5-HT7R, was found to have an angiogenic effect *in vitro* and *in vivo*. **Conclusion:** The 5-HT7Rs can be considered to have an important role in EC functions and angiogenesis. Thus, 5-HT7Rs may be a potential target for the therapeutic interventions aimed to regulate the process of angiogenesis in diseases such as varicose veins, peripheral vascular diseases or vasculature of solid tumors. The LP-211 may be a candidate to improve the ineffective circulation in related disorders.

Key words: Angiogenesis,5-hydroxytriptamine type 7 receptor, LP-211, serotonin, endothelial cells

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Corresponding Author: Fatih Yesildal, Department of Medical Biochemistry, Istanbul Medeniyet University, Goztepe Education and Research Hospital, Kadikoy, 34722 Istanbul, Turkey Tel: +902165709023 Fax: +902165666614

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Serotonin (5-hydroxytriptamine; 5-HT) is a well-known neurotransmitter functioning in regulation of many physiological and pathological pathways in the organism[1]. Serotonin shows its effects via more than a dozen of membrane receptors which are classified in seven main families according to their structural and operational features[2]. 5-HT7 receptor (5-HT7R) was the last receptor family defined and cloned, belonging to serotonin receptors[3]. The 5-HT7Rs are found not only in central nervous system (CNS) (commonly in thalamus, hippocampus, cortex), but also in peripheral tissues mainly in vessels and intestines[3-5]. Targeting of the 5-HT7Rs in some animal studies have implied that these receptors had a key role in many situations in a living organism. Some of the situations, in which serotonin and 5-HT7Rs are involved, include memory and learning[6], regulation of body temperature[7,8], circadian rhythm and sleep[3], migraine[9] and anxiety[10].

There are many studies defining the agonists and antagonists of 5HT7Rs[11]. The LP-211 [N-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide] was reported to be a selective agonist of 5-HT7R with a high affinity[11].

Angiogenesis is the generation of vascular network by endothelial cells from existing vasculature and is composed of some phases including proliferation, migration, maturation and morphogenesis[12,13]. It is requisite during the life span, beginning with embryonic development and going on in many physiological situations such as growth, wound healing and regeneration of tissues[14]. Angiogenesis plays a key role in pathogenesis of many commonly seen diseases as well; such as cancer, macular degeneration, chronic inflammatory diseases[15]. Thus, many studies have been conducted to understand how angiogenesis is regulated[14,15].

5-HT promotes angiogenesis via inducing endothelial cell (EC) proliferation and migration[16,17]. Additionally, 5-HT was implied to have an angiogenic function in colon cancer growth by decreasing the expression of matrix metalloproteinase 12 resulting in a decline in production of angiostatin which is known as an anti-angiogenic peptide[18]. In a study, the presence of 5-HT7R was demonstrated in ECs[19] and 5-HT4R was found to regulate angiogenesis[20]. However, there is no data about the effect of selective agonists of 5-HT7Rs on angiogenesis.

The aim of this study was to investigate whether 5-HT7R stimulation by its selective agonist LP-211 promoted angiogenesis. To our knowledge, this is the first study showing the effect of LP-211, the selective agonist of 5-HT7R, on angiogenesis.

MATERIALS AND METHODS

This study was carried out in Vascular Biology Laboratory which is located in Gulhane Education and Research Hospital Department of Medical Biochemistry, between June, 2015 and September, 2017.

LP-211 solution: The 6 mM of LP-211 [N-(4-cyanophenyl) methyl)-4-(2-diphenyl)-1-piperazinehexanamide; $C_{30}H_{34}N_4O$] (Sigma-Aldrich Chemie GmbH, Germany) stock solution was used for serial dilutions to obtain the required concentrations for *in vitro* and *in vivo* assays of angiogenesis. LP-211 was dissolved in dimethyl sulfoxide (DMSO). Therefore, DMSO was used in control groups of the *in vitro* and *in vivo* angiogenesis assays.

Cell proliferation assay: The proliferative effect of LP-211 on Human Umbilical Vein Endothelial Cells (HUVECs) was determined, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method by following the same steps in previous study by Yesildal *et al.*[21]. Briefly, each well containing approximately 20000 HUVECs were treated with LP-211 and vehicle (control). The LP-211 solutions with various concentrations (100, 250, 500 and 1000 nM) were used to see the effect of LP-211 on HUVECs after an incubation period of 24-48 h. Absorbance of LP-211 treated wells were compared with control wells. Cell proliferation of LP-211 treated wells were expressed in terms of percent (%) of control wells.

***In vitro* endothelial cell tube formation assay:** Matrigel (BD cat. no.: 354234, USA) assay was performed by using 0.5 μM LP-211 solution. The change in tube formations with respect to the control group was evaluated according to the tube length/area ratio. Each phase in this assay was well-defined in published study by Yesildal *et al.*[21]. Briefly, HUVECs were placed on each well containing Matrigel, at a density of 10000 cells/well and treated with LP-211 and vehicle. After 18 h of incubation, tube formations were evaluated under light microscope.

Chick chorioallantoic membrane (CAM) assay: CAM assay was employed to see the *in vivo* effects of LP-211 on angiogenesis. About 10, 25 and 50 μM of LP-211 solutions were used in CAM assay. All the procedures in CAM assay were performed as described step by step in previously published study by Yesildal *et al.*[21]. Briefly, a window was opened on each of the fertilized chicken eggs after an incubation period of six days. LP-211 and vehicle solutions were placed on CAM

area of each egg. On seventh day, each CAM was photographed and efficacy of LP-211 on CAM model was scored.

Statistical analysis: MedCalc software for Windows, Version 9.2.0.1 (MedCalc, Belgium) was used for statistical analysis. The limit of 0.05 for p value was used as an indicator of statistically significance. Values were given as Mean±standard deviation.

RESULTS

Cell proliferation assay of LP-211: According to the results of MTT assay, LP-211 showed proliferative effects on endothelial cells. After an incubation period of 24 h and 48 h, the proliferation in LP-211 treated wells was found to be significantly higher with respect to the control wells ($p<0.05$) (Fig. 1).

Effect of LP-211 on tube formation: The tube formations in LP-211 treated group were much more with respect to the control group (Fig. 2). Considering the tube length/area ratio, LP-211 treated group had significantly higher results according to the control group; after 18 h of incubation ($p<0.05$) (Fig. 3). These results provide evidence that LP-211 had angiogenic effects *in vitro*.

LP-211 induces angiogenesis on CAM: Formation of new vessels was macroscopically evaluated and scored on CAM area and the results were given in Table 1. Proangiogenic effects of indicated concentrations of LP-211 were compared by chi square test. Angiogenic effect of LP-211 was found to be dose-dependent. The difference between doses was

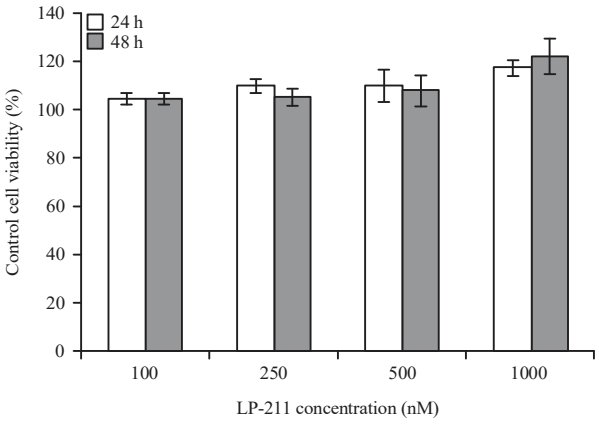

Fig. 1: LP-211 showed a proliferative effect on HUVECs
Especially 500 nM or greater doses caused a significant proliferation of HUVECs compared to the control group, cell proliferation of LP-211 treated wells were shown as control group (%), (control wells were considered as 100%)

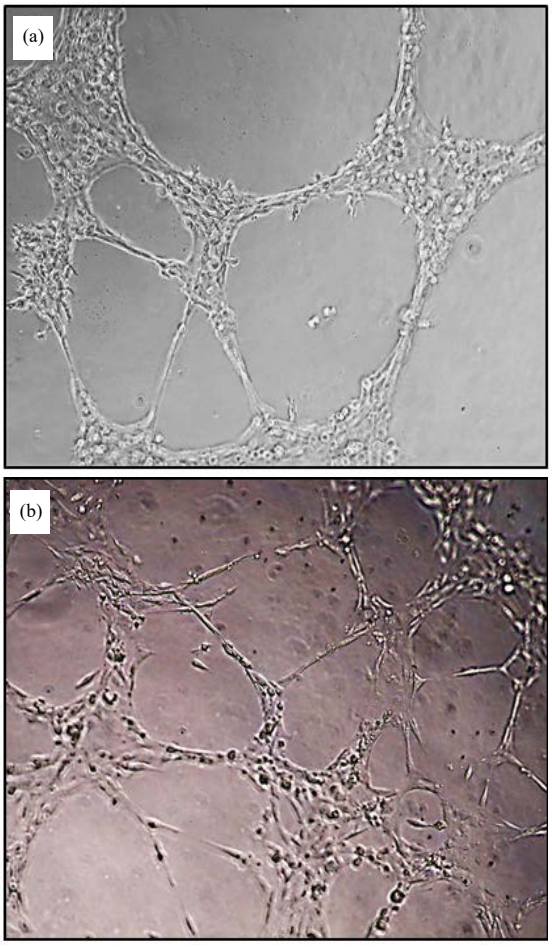

Fig. 2(a-b): Tube formations (X40 magnification) in (a) Control and (b) LP-211 treated wells
Number of tube formations was greater in LP-211 treated wells than that of control group

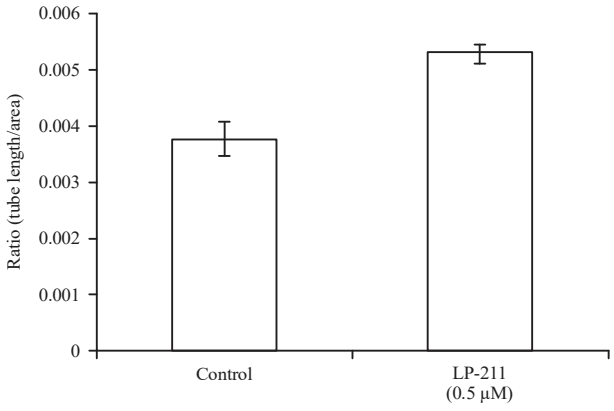

Fig. 3: LP-211 treated wells (0.5 µM) resulted in higher levels of tube length/area ratio
Ratio of the length of tube like structures to their area was calculated for each control and LP-211 treated well to make an objective evaluation of the tube formation assay

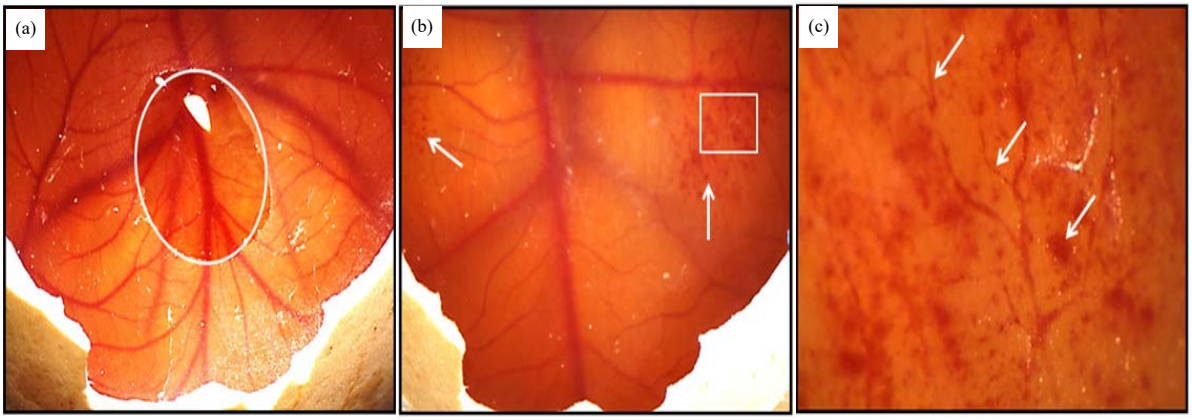

Fig. 4(a-c): Effect of LP-211 on CAM (a) Before and (b-c) After 24 h
a, b (X1.0 magnification), c (white rectangle in b was magnified X4.0), a: White circular part was the place where LP-211 was applied first, b: LP-211 showed angiogenic effect on CAM area, white arrows indicate extravasation and sprouting of new vessels, CAM: chorioallantoic membrane

Table 1: Efficacy of LP-211 on CAM vasculature

	Efficacy							
	Ineffective		+1		+2		Total	
Groups	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Control	6	100.00	0	0.00	0	0.00	6	100
10 µM	1	20.00	3	60.00	1	20.00	5	100
25 µM	0	0.00	4	80.00	1	20.00	5	100
50 µM	0	0.00	3	50.00	3	50.00	6	100
Total	7	31.82	10	45.45	5	22.73	22	100

A 0, 10, 25 and 50 µM concentrations of LP-211 were placed on CAM area and their angiogenic effects were scored, "Ineffective" response indicates no obvious change in CAM area, only the physiological growth, "+1 score" indicates sprouting of a few new vessels in CAM area, "+2 score" indicates budding and sprouting of many new vessels in CAM area, vessel density and number of branching points are much greater, as the administered LP-211 concentration increased, angiogenic effect increased, even 10 µM of LP-211 showed an increase in CAM area of 80% of the used eggs, 50 µM of LP-211 produced a marked increase in all CAMs (100%), at this dose, LP-211 caused extravasation and sprouting at many points of the present vessels and vessel density in CAM area was the highest of all

statistically significant (Pearson chi square = 20.1, p = 0.003) and also a strong correlation was found between LP-211 concentrations and proangiogenic efficacies (Spearman's correlation r = 0.764, p<0.001). When treated with LP-211, a significant increase (extravasation and sprouting) in CAM vasculature was seen (Fig. 4). These results indicate that LP-211 had angiogenic effects *in vivo*.

DISCUSSION

Information about 5HT7R is getting clearer as modern molecular biology and traditional medical chemistry and pharmacology is combined. Over the last two decades, varios selective agonists of 5HT7Rs have been identified and LP-211 is one of them[22]. The LP-211 is likely to have both suitable pharmacokinetic and pharmacodynamic features. For example, its half life is longer unlike the rapidly metabolized LP-44 *in vivo*[23]. Thus, LP-211 was selected in this study to investigate how 5-HT7R stimulation affect angiogenesis.

In this study, both *in vitro* and *in vivo* assays for angiogenesis were used. Cell proliferation assay showed that LP-211 promoted proliferation of ECs *in vitro*. Matrigel assay is a specific model for angiogenesis in which the potential of ECs to create three-dimensional tube structures in the basement membrane is investigated. Activation of 5-HT7Rs on ECs by LP-211 resulted in migration of ECs and formation of tubes which provided evidence that LP-211 induced angiogenesis *in vitro*. In CAM assay, likely to the *in vitro* results, LP-211 was found to boost angiogenic response by sprouting of new vessels. Increase of vascular permeability is considered to be an indicator of angiogenic response in many conditions such as tumors and wound healing[24,25]. In CAM assay, extravasation was apparently seen on CAMs which were treated with LP-211. These results indicated that LP-211 had an angiogenic effect *in vivo*.

It was reported that 5-HT4 receptor stimulated angiogenesis[20]. On the other hand, 5-HT4 receptor agonist mosapride was found to have anti-angiogenic effect

in vitro[26]. ECs were shown to express 5-HT7Rs in a study[19]. In another study, endogenously synthesized 5-HT7R was found to induce EC migration[27]. Therefore, 5-HT receptors are considered to have a role on regulation of angiogenesis. On the contrary, another study showed that 5-HT7Rs were not involved in angiogenic effect of 5-HT[28].

According to these data, serotonin and its receptors have a distinct role in angiogenic pathways. This study provided evidence that stimulation of 5-HT7Rs with their selective agonist LP-211 boosted angiogenic activity. However, molecular pathways of this effect is still unclear. The limitation of this study is the lack of an animal model. Despite CAM assay is an *in vivo* angiogenesis model, these data should be confirmed with animal experiments, such as wound healing model or hind limb ischemia model. On the other hand, serotonin and its receptors have a wide range of effects in living organisms. So, stimulation of 5-HT7Rs may affect many other systems. Nevertheless, targeting of 5-HT7Rs topically, seems to help solving many problems about insufficient circulation and insufficient vasculature.

CONCLUSION

In conclusion, 5-HT7 receptor activation by its selective agonist LP-211 resulted in an increase in angiogenic response. According to these results, 5-HT7Rs can be considered to have an important role in EC functions and angiogenesis. The LP-211 may be a candidate to improve the ineffective circulation in related disorders. Further studies should be performed to investigate whether 5-HT7Rs had a relation with VEGF signaling pathway and whether this angiogenic effect was available for all other 5-HT receptors.

SIGNIFICANCE STATEMENT

This study discovers the angiogenic effect of LP-211, a selective agonist of 5-HT7Rs, that can be beneficial for wound healing, peripheral vascular diseases and such conditions in which vasculature and blood circulation is insufficient. This study will help the researcher to uncover the critical area of targeting serotonin receptors in "angiogenesis related diseases" that many researchers were not able to explore. Thus a new theory on 5-HT7Rs in angiogenic pathways may be arrived at.

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