Department of Stomatology, Beijing Tongren Hospital Affiliated to Capital Medical University, Bejing, China

A combination of irsogladine maleate and azithromycin exhibits addictive protective effects in LPS-induced human gingival epithelial cells

Xiaodong Feng, Jingming Liu*

Received June 6, 2013, accepted October 7, 2016

*Corresponding author: Jingming Liu, Department of Stomatology, Beijing Tongren Hospital Affiliated to Capital Medical University, No.1 Dongjiaomin Lane, Dongcheng District, Beijing 100730, China
liujingming1001@126.com

Pharmazie 72: 91–94 (2017) *doi: 10.1691/ph.2017.6697*

Objective: This study aimed to investigate the potential effects of the combination therapy of irsogladine maleate (IM) and azithromycin (AZM) on the inflammation in lipopolysaccharide (LPS)-induced gingival epithelial cells. *Methods:* Human gingival epithelial cell OBA-9 was stimulated by LPS to construct the periodontitis model, followed by the treatment of irsogladine maleate (IM) or azithromycin (AZM) with different concentration. Trans-epithelial electrical resistance (TER) of cells in each group was analyzed, and qRT-PCR and western blotting were used to detect the expressions of inflammatory cytokines. Immunofluorescence staining was performed to detect the protein expression. *Results:* The TER for cells was significantly decreased while the inflammatory cytokines expressions including IL-6, IL-8, IL-1β and TNF-α were all significantly increased by LPS compared to the control (P<0.05). However, TER was increased significantly, whereas the cytokine levels were decreased by IM or AZM, but these effects was more apparent in cells treated with IM and AZM combination (P<0.01). Moreover, E-cadherin and vimentin expressions were more positive in the IM and AZM group than in the other groups. The application of ERK and P38 MAPK inhibitors reversed the effects of LPS on cell inflammatory cytokine production and cell TER. *Conclusion:* This study revealed that the combination therapy of IM and AZM performed excellent effects on preventing the inflammatory progression of periodontitis.

1. Introduction

Periodontitis is a common disease characterized by symptoms like gum inflammation and swelling, bleeding, alveolar bone resorption, periodontal pocket formation and tooth mobility (Bullon et al. 2014). The pathogenesis of periodontitis is complicated, and immune reactions, inflammatory reactions and pathogenic bacteria including helix melanin producing *Bacteroides* and *Actinomycetes* species are involved (Ishikawa et al. 1997). To date, periodontitis treatment includes systemic and local administration and local drug administration and fundamental dental plaque treatment (Borole et al. 2014; Manetsch et al. 2014). However, treatment outcomes still remain unsatisfactory due to the complicated pathogenesis of the disease.

Irsogladine maleate (IM) has been reported to be a useful drug in the clinical cure of gastric ulcer through protecting the mucosa (Hiraishi et al. 2010). Recent studies have reported that IM could suppress the secretion of cytokines and gingival epithelial cell adhesion induced by *Actinobacillus*, and protect the oral mucosa (Uchida et al. 2002; Chahboun et al. 2014). Azithromycin (AZM), a macrolide antibiotic, has been reported to function as an anti-inflammatory agent through anti-infection and immune regulation in various of diseases (Bakheit et al. 2014; Havlir et al. 1996). *In vitro* studies revealed that AZM could suppress the secretion of inflammatory cytokines (Doyle et al. 2015; Shunmugaperumal and Kaur 2015). Few studies have mentioned the potential effects of a combination of IM and AZM in human periodontitis therapy.

In the current study, we constructed a periodontitis model *in vitro* by stimulating the human gingival epithelial cell line OBA-9 using lipopolysaccharide (LPS), and then analyzed the influences of IM and AZM on transepithelial electrical resistance (TER) and inflammatory cytokine secretion. This study aimed to investigate the effects of a combination therapy of IM and AZM in the cure of periodontitis and to reveal its potential molecular mechanisms.

2. Investigations and results

2.1. Combination of IM and AZM enhanced TER

To analyze the effect of combination therapy of IM and AZM on the inflammations in cells, the transepithelial electrical resistance (TER) was measured (Fig. 1). LPS treatment significantly reduced the cell TER compared to the controls with time increasing (P<0.001), the TER was partly increased by the single treatment of IM or AZM (P<0.05). Interestingly, cell TER was highly increased up to values close to control by the combination treatment of IM and AZM.

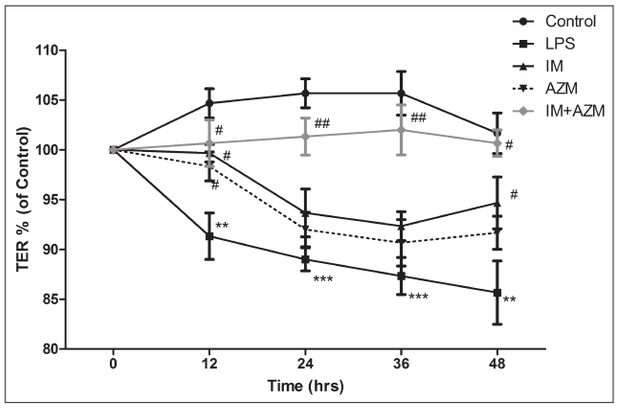

Fig. 1: Combination therapy of IM and AZM enhanced the cell transepithelial electrical resistance (TER). Cell TER was significantly decreased by LPS, but was partly increased by the IM or AZM treatment, however, TER was significantly increased by the combination of IM and AZM compared to the LSP group, which was almost the same as control. **: P<0.01, and **: P<0.001 compared to the control; #: P<0.05, ##: P<0.01, and ###: P<0.001 compared to the LSP treated group.

2.2. Combination of IM and AZM reduced inflammatory cytokine secretion

We further analyzed the effects of combination therapy of IM and AZM on the inflammatory cytokine secretion (Fig. 2). Cytokine levels including those of IL-6, IL-8, IL-1β and TNF-α were all significantly increased by the LPS treatment (P<0.05), whereas, they were significantly decreased by the combination therapy of IM and AZM compared to the single treatment of IM or AZM (P<0.01).

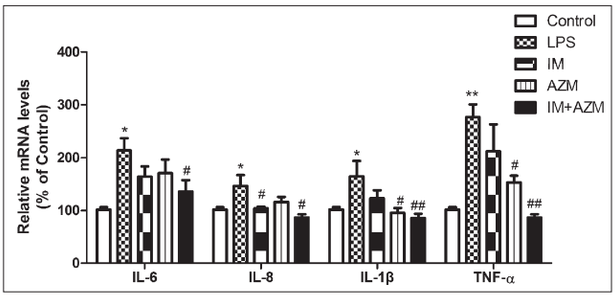

Fig. 2: Combination therapy of IM and AZM reduced the inflammatory cytokine secretion in cells. Cytokines including IL-6, IL-8, IL-1β and TNF-α were all highly expressed in cells treated with LPS, however, their levels were significantly reduced by the combination therapy of IM and AZM. **: P<0.01, and **: P<0.001 compared to the control; #: P<0.05, and ##: P<0.01 compared to the LSP treated group.

2.3. Combination of IM and AZM increased the expressions of E-cadherin and vimentin

To analyze the potential effect of IM and AZM combination from a molecular perspective, the expression of vimentin and E-cadherin was analyzed. Immunofluorescence assay showed that vimentin and E-cadherin expressions were drastically decreased in the LPS treated group compared to the controls (Fig. 3). Additionally, expression of the two proteins were highly expressed by the combination therapy of IM and AZM compared to that in cells treated with IM or AZM alone.

2.4. Combination of IM and AZM activated the ERK/P38-AMPK signal pathway

The phosphorylated (p-)p38 and p-ERK were highly expressed by the LPS inducement, but was decreased by the single IM or AZM treatment. However, the expressions of p-ERK and p-AMPK were drastically decreased by the combination therapy (Fig. 4). These results indicated that the p38 AMPK/ERK signal pathway could be deactivated by the treatment of IM and AZM.

2.5. The reverse test in vitro

To further analyze the potential roles of IM and AZM combination therapy on the ERK/AMPK signal pathway-related protein expression, the AMPK or ERK inhibitor was introduced to the study (Fig. 5). SB203580 and PD98059 showed to inhibit AMPK and ERK in previous studies (Clerk and Sugden 1998; Yamaguchi et al. 2002). When cells were treated with the inhibitors, the percentage of TER was significantly increased compared to the LPS treatment group with time increasing (P<0.01, Fig. 5A). In addition, the secretion of cytokines including IL-6, IL-8, IL-1β and TNF-α was significantly decreased by the inhibitor application compared to the LPS treated group (P<0.01, Fig. 5B).

3. Discussion

Periodontitis remains to be a common oral disease, which is characterized by high morbidity and may become a striking health problem (Darveau 2010). The pathogenesis of periodontitis includes bacterial infections, which are difficult to treat (Tonetti et al. 2007; Hajishengallis 2014). In this study, we tried to develop a novel therapy method for periodontitis by using two anti-inflammatory drugs, IM and AZM, and to analyze possible molecular mechanism for an effective combination therapy.

Studies have revealed that TER represents the permeability of a cell membrane for inside and outside ions (Min and Min 2013). Inflammatory infections may decrease the TER of gingival cells (Blascobaque et al. 2012), while IM or AZM treatment could both increase the TER of gingival cells (Kishimoto et al. 2008; Oteo et al. 2010), which was in accordance with our results (Fig. 1). However, our data revealed that the combination therapy of IM and AZM could excellently increase the TER, indicating that a combination therapy of these drugs may be useful in the treatment of periodontitis. In addition, we further detected the inflammatory cytokines in each group. Savitri et al. (2014) proved that IM inhibited the *Porphyromonas gingivalis*-mediated expression of IL-8 and then played certain roles in preventing the progression of periodontitis, whereas Doyle et al. (2015) demonstrated that AZM suppressed the production of pro-inflammatory cytokines and chemokines in human gingival fibroblasts. Our results showed that the LPS treatment resulted in a high expression of IL-6, IL-8, IL-1β, and TNF-α, while their levels were decreased by the single treatment of IM or AZM at different levels, namely, IL-8 and TNF-α depression by IM, and IL-6 and IL-1β depression by AZM. Interestingly, their levels can be significantly decreased by the combination therapy of IM and AZM (Fig. 2), indicating that excellent effects of the IM and AZM combination on alleviating the inflammatory in cells.

It has been well known that E-cadherin and vimentin may serve as indicators of epithelial-mesenchymal transition (Myong 2012). Inflammatory cytokines or chemokines would destroy the gingival

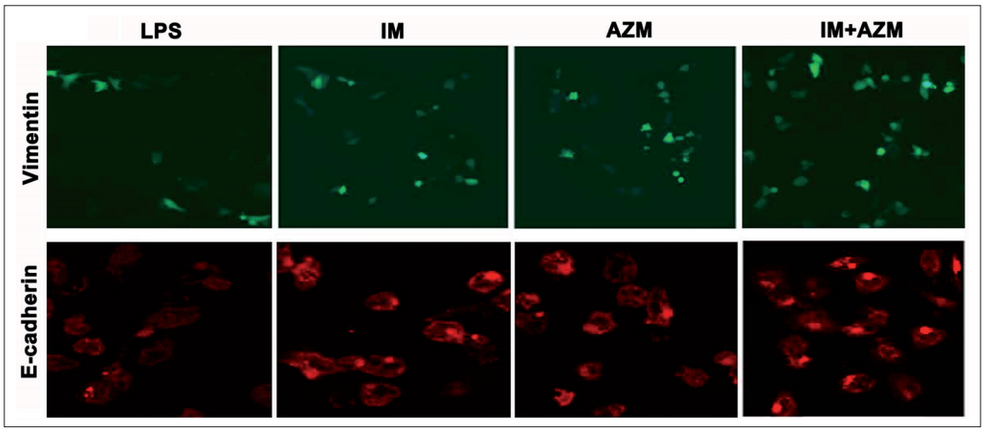

Fig. 3: Combination therapy of IM and AZM increased the expression of Vimentin and E-cadherin. Immunofluorescence assay showed that Vimentin and E-cadherin expressions were drastically decreased in LPS treated group compared to the control group, while expression of the two kinds of proteins were highly expressed by the combination therapy of IM and AZM compared to that in cells treated with single IM or AZM.

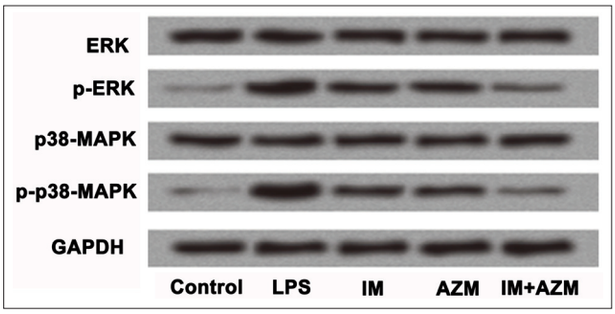

Fig. 4: Combination therapy of IM and AZM activated the ERK/AMPK signal pathway. P-ERK and p-AMPK protein were highly expressed in LPS treated group, while they were slowly expressed by the signal treatment of IM or AZM, however, their expression was even lowly expressed by the combination therapy of IM and AZM.

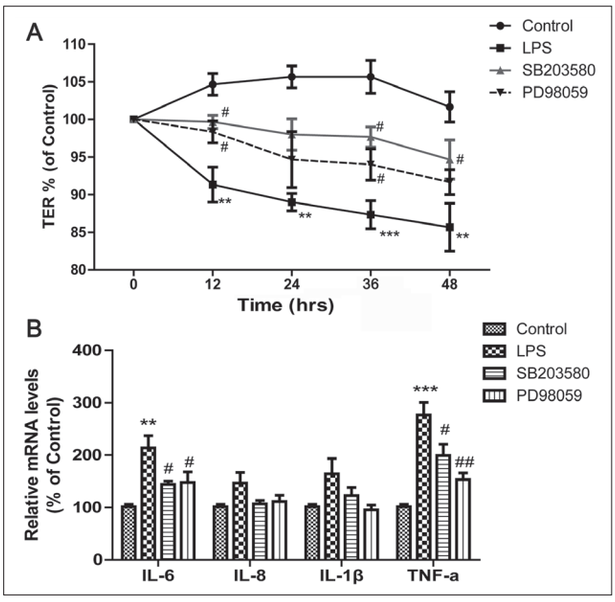

Fig. 5: Inhibitor of ERK/AMPK signal pathway reversed the expression of TER and cytokine secretion caused by LPS in cells. A: the inhibitor application of SB203580 or PD98059 enhanced the percentage of TER in cells compared to the LPS treated group; B: secretion of IL-6, IL-8, IL-1β and TNF-α was reduced by the inhibitor treatment of SB203580 or PD98059 compared to the LPS-treated group. **: P<0.01, and ***: P<0.001 compared to the control; #: P<0.05, and ##: P<0.01 compared to the LSP treated group.

tissue through affecting the epithelial-mesenchymal transition. Our data showed that the inflammatory of LPS stimulation resulted in â decrease of vimentin and E-cadherin in cells, while the two proteins were higher expressed in cells treated with IM or AZM than the control (Fig. 3). We hypothesized that IM and AZM combination treatment would promote the epithelial-mesenchymal transition in gingival cells.

Furthermore, p38 and ERK are pivotal cell transduction signals, which are involved in various processes including cell proliferation, apoptosis, and inflammation (Bordicchia et al. 2012; Ma et al. 2014). Previous studies suggested a correlation between p38 or ERK signal and periodontitis, but no evidence has proved the effects of IM or AZM on p38 or ERK signals in human gingival cells. Rogers et al. (2007) had demonstrated that the p38 MAPK inhibitor could arrest bone loss during periodontitis development in a rat model, and Yoshimoto et al. (2015) proved that the ERK signal was involved in TGF-β1 induced cell apoptosis in gingival epithelial cells. Since SB203580 and PD98059 are inhibitors for AMPK and ERK (Clerk and Sugden 1998; Yamaguchi et al. 2002), we evaluated the effects of IM and AZM on inflammatory cytokine secretion and TER of gingival cells using the two inhibitors. In agreement with previous results (Blascobaque et al. 2012), our results showed that the ERK and p38 MAPK signal were both activated by the application of LPS in gingival cells. However, the reverse validate results revealed that the inflammatory reaction was suppressed while cell TER was increased by the application of ERK and p38 MAPK inhibitor (Fig. 4), suggesting a suppression of IM and AZM combination on the activation of the ERK/P38 MAPK signal.

To sum up, the data presented in this study reveals that a combination therapy of IM and AZM plays a role in the development and progression of periodontitis via suppressing the inflammatory reaction and promoting epithelial-mesenchymal transition through inhibiting the activation of the ERK/P38 MAPK signal pathway. This study may provide the theoretical basis for an improved treatment of periodontitis, but further studies are needed to investigate mechanism in detail andf to implement this approach in clinical practice.

4. Experimental

4.1. Cell culture

The immortalized human gingival epithelial cell OBA-9 (HGEC) line (obtained from ATCC) was cultured in Humedia-KG2 medium (Kurabo, Osaka, Japan) containing insulin (10 mg/mL), human epidermal growth factor (0.1 mg/mL), hydrocortisone (0.67 mg/mL), bovine pituitary extract (150 mg/mL), gentamycin (100 mg/mL) and amphotericin B (100 mg/mL). The cells were incubated at 37 °C in an atmosphere of 5% CO_2 until they reached confluence.

4.2. Trans-epithelial electrical resistance (TER)

Cells were seeded at a density of 8×10^4 cells/mL on the polyethylene terephthalate membrane of a cell culture insert, and maintained in 800 mL of medium A (insert: 300 mL, lower chamber: 500 ml). Confluent HGEC were pretreated with DMSO, irsogladine maleate (IM, 1 M), azithromycin (AZM, 60 mg/mL), irsogladine maleate + AZM (IM+AZM), SB203580 (10 M, a p38 MAP kinase inhibitor) and PD98059 (10 M, an ERK inhibitor) for 1 h. They were then exposed to LPS at 1 g/ mL for 24 h at 37 °C in a 5% CO_2 atmosphere in serum-free medium. TER of HGEC was measured using a Millicell-ERS (Millipore, Billerica, MA, USA).

4.3. Real-time PCR

To analyze cytokines mRNA, confluent HGEC were pretreated with DMSO, irsogladine maleate (IM, 1 μM), azithromycin (AZM, 60 mg/ml), irsogladine maleate + AZM (IM+AZM), SB203580 (10 μM, a p38 MAP kinase inhibitor) and PD98059 (10 μM, an ERK inhibitor) for 1 h. They were then exposed to LPS at 1 mg/mL for 24 h at 37 °C in a 5% CO_2 atmosphere in serum-free medium. Total RNA was extracted using TRIZOL reagent and assessed for quantity and integrity using agarose gel electrophoresis and Eppendorf Biophotometer Plus. Total mRNA (1 μg) was reverse transcribed using PrimeScript@ 1-st Strand cDNA Synthesis Kit according to the manufacturer's manual. Real-time PCR was performed on a fluorescence ration PCR instrument using SYBR@ Premix Ex TaqTM II. Target cDNA (TNF-α, IL-6, IL-8, IL-1β) and endogenous control cDNA (β-actin) were amplified under the following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. Relative quantitative (RQ) measurements of target gene levels were performed using the ΔΔCt method, where Ct is the threshold concentration.

4.4. Immunofluorescence

Immunofluorescence staining was performed using rabbit anti-mouse E-cadherin (Santa Cruz Biotechnology, Inc.), vimentin (Cell Signaling Technology) and β-actin (Santa Cruz Biotechnology Inc.) as previously described. In brief, HGEC cells were cultured on 10-mm round coverslips and stained using standard methods. Cells were mounted on slides using ProlongH Gold antifade reagent (Life Technologies Corporation, Carlsbad, CA, USA) and imaged by fluorescence microscopy (Olympus).

4.5. Western Blot

Cells were pretreated with DMSO, irsogladine maleate (IM, 1mM), azithromycin (AZM, 60 mg/mL) and irsogladine maleate + AZM (IM+AZM) for 1 h. The cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100) containing protease and phosphatase inhibitor. Cell lysate protein content was determined using a bicinchoninic acid (BCA) protein assay kit. Equivalent amounts of whole cell extracts were subjected to SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 2 h and then incubated with respective primary antibody overnight at 4 °C followed by the incubation with the appropriate HRP-conjugated secondary antibody for 1.5 h at room temperature. Blots were visualized with an ECL detection kit (Pierce, USA) and analyzed using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, USA).

4.6. Statistical analysis

Data are expressed as the mean ± SEM. The data were analyzed using Student's t test or the ANOVA test. Results with $P<0.05$ were considered statistically significant. GraphPad Prism (GraphPad Software Inc., San Diego, California, USA) was used for these analyses.

Conflicts of interest: None declared.

References

Bakheit AHH, Al-Hadiya BMH, Abd-Elgalil AA (2014) Azithromycin. In: Profiles of Drug Substances, Excipients and related Methodology 39: 1-40.

Blascobaque V, Serino M, Vergnes JN, Riant E, Loubieres P, Arnal JF, Gourdy P, Sixou M, Burcelin R, Kemoun P (2012) High-fat diet induces periodontitis in mice through lipopolysaccharides (LPS) receptor signaling: protective action of estrogens. Plos One 7: e48220-e48220.

Bordicchia M, Liu D, Amri EZ, Ailhaud G, Dessì-Fulgheri P, Zhang C, Takahashi N, Sarzani R, Collins S (2012) Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. J Clin Invest 122: 1022-1036.

Borole DK, Sriharsha SN, Yashwant KD, Neelima KSSN (2014) Preparation and characterization of metronidazole and minocycline HCl mucoadhesive gel for the treatment of periodontitis. Int J Chem Pharm Sci 2: 1344-1350.

Bullon P, Newman HN, Battino M (2014) Obesity, diabetes mellitus, atherosclerosis and chronic periodontitis: a shared pathology via oxidative stress and mitochondrial dysfunction? Periodontology 2000 64: 139-153.

Chahboun H, Arnau MM, Herrera D, Sanz M, Ennibi OK (2014) Bacterial profile of aggressive periodontitis in Morocco: a cross-sectional study. BMC Oral Health 15: 1-8.

Clerk A, Sugden PH (1998) The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs). FEBS Lett 426: 93-96.

Darveau RP (2010) Periodontitis: a polymicrobial disruption of host homeostasis. Nature Rev Microbiol 8: 481-490.

Doyle CJ, Fitzsimmons TR, Marchant C, Dharmapatni AASSK, Hirsch R, Bartold PM (2015) Azithromycin suppresses P. gingivalis LPS-induced pro-inflammatory cytokine and chemokine production by human gingival fibroblasts in vitro. Clin Oral Invest 19: 221-227.

Hajishengallis G (2014) Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response: Trends Immunol 35: 3-11.

Havlir DV, Dubé MP, Sattler FR, Forthal DN, Kemper CA, Dunne MW, Parenti DM, Lavelle JP, White AC Jr, Witt MD, Bozzette SA, McCutchan JA (1996) Prophylaxis against disseminated Mycobacterium avium complex with weekly azithromycin, daily rifabutin, or both. New Engl J Med 335: 392-398.

Hiraishi H, Haruma K, Miwa H, Goto H (2010) Clinical trial: irsogladine maleate, a mucosal protective drug, accelerates gastric ulcer healing after treatment for eradication of Helicobacter pylori infection – the results of a multicentre, double-blind, randomized clinical trial (IMPACT study). Alim Pharmacol Ther 31: 824-833.

Ishikawa I, Nakashima K, Koseki T, Nagasawa T, Watanabe H, Arakawa S, Nitta H, Nishihara T (1997) Induction of the immune response to periodontopathic bacteria and its role in the pathogenesis of periodontitis. Periodontology 14: 79–111.

Kishimoto A, Fujita T, Shiba H, Hayashida K, Uchida Y, Kawaguchi H, Kurihara H (2008) Irsogladine maleate suppressed infiltration of inflammatory cells in a rat periodontitis model. Program and Abstracts of Annual Meeting of the Japanese Society of Periodontology, pp 39.

Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue TL (2014) Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. Diabetes 63: 3324-3333.

Manetsch R, Shaw LN, Van Horn KS, Burda WN (2014) Compositions, methods of use, and methods of treatment. US, 13/848846.

Min C, Min C (2013) Study between periodontitis and premature rupture of membrane at gestation. Abstract, 10th World Congress on Preventive Dentistry.

Myong NH (2012) Loss of E-cadherin and acquisition of vimentin in epithelial-mesenchymal transition are noble indicators of uterine cervix cancer progression. Cerebral Cortex 46: 341-348.

Oteo A, Herrera D, Figuero E, O'Connor A, González I, Sanz M (2010) Azithromycin as an adjunct to scaling and root planing in the treatment of Porphyromonas gingivalis-associated periodontitis: a pilot study. J Clin Periodontol 37: 1005-1015.

Rogers JE, Li F, Coatney DD, Otremba J, Kriegl JM, Protter TA, Higgins LS, Medicherla S, Kirkwood KL (2007) A p38 mitogen-activated protein kinase inhibitor arrests active alveolar bone loss in a rat periodontitis model. J Periodontol 78: 1992-1998.

Savitri IJ, Ouhara K, Fujita T, Kajiya M, Miyagawa T, Kittaka M, Yamakawa M, Shiba H, Kurihara H (2014) Irsogladine maleate inhibits Porphyromonas gingivalis-mediated expression of toll-like receptor 2 and interleukin-8 in human gingival epithelial cells. J Periodont Res 50: 486-493.

Shunmugaperumal T, Kaur V (2015) In vitro anti-inflammatory and antimicrobial activities of azithromycin after loaded in chitosan- and Tween 20-based oil-in-water macroemulsion for acne management. Aaps Pharmscitech 1-10.

Tonetti MS, D'Aiuto F, Nibali L, Donald A, Storry C, Parkar M, Suvan J, Hingorani AD, Vallance P, Deanfield J (2007) Treatment of periodontitis and endothelial function. New Engl J Med 356: 911-920.

Uchida Y, Shiba H, Komatsuzawa H, Noguchi T, Ogawa T, Kawaguchi H, Sugai M, Kurihara H (2002) Effects of irsogladine maleate on human gingival epithelial cells challenged with Actinobacillus actinomycetemcomitans. J Jap Assoc Periodontol 44:106.

Wehbe M, Soudja SM, Mas A, Chasson L, Guinamard R, de Tenbossche CP, Verdeil G, Van den Eynde B, Schmitt-Verhulst AM (2012) Epithelial-mesenchymal-transition-like and TGFβ pathways associated with autochthonous inflammatory melanoma development in mice. Plos One 7: e49419.

Yamaguchi K, Tomita H, Sugano E, Nakazawa T, Tamai M (2002) Mitogen-activated protein kinase inhibitor, PD98059, inhibits rat retinal pigment epithelial cell replication by cell cycle arrest. Jap J Ophthalmol 46: 634-639.

Yoshimoto T, Fujita T, Kajiya M, Matsuda S, Ouhara K, Shiba H, Kurihara H (2015) Involvement of smad2 and Erk/Akt cascade in TGF-β1-induced apoptosis in human gingival epithelial cells. Cytokine 75: 165-173.