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Genotoxic and cytotoxic assessment of sitagliptin and simvastatin alone and in combination

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Abstract: Type 2 Diabetes Mellitus (T2DM) patients are at high risk of Coronary Heart Disease (CHD) and need a global therapeutic intervention. A fixed-dose combination prescription medication containing anti-diabetic drug (Sitagliptin) and lipid lowering (Simvastatin) has recently been approved. Present study was designed to explore the potential synergistic toxic effects of sitagliptin and simvastatin at cellular level. MTT assay revealed the potential synergistic cytotoxic effect whereas Comet assay spotlighted the genotoxicity. MTT assay conducted on Vero cell lines revealed no significant change in proliferative activity upon treatment with simvastatin but cell survival percentage (CSP) decreased upon treatment with sitagliptin (51% at 1000µg/mL). However, combination of both drugs exhibited a better survival percentage except highest dose combination (1000:500µg/mL) which augmented antiproliferative effects rendering CSP 71.6%. The genotoxic assay spotted that Simvastatin produced less damage to DNA with the threshold of 500µg/ml whereas Sitagliptin significantly damage above the 250µg/mL, However, combination of drugs produced lesser damage than Sitagliptin alone. The findings concluded a non-genotoxic combination of sitagliptin and simvastatin which possess a least cytotoxic potential suggesting the safe use of the combination both in T2DM and CHD.

Keywords: Sitagliptin, simvastatin, Comet assay, MTT assay.

INTRODUCTION

Understanding the role of type-2 diabetes (T2D) in the pathogenesis of coronary heart disease (CHD) is a fundamental problem for the design of effective approaches for preventing cardiovascular disease. T2D is associated with an increased risk of CHD by two to fourfold in observational studies (Ahmad *et al*., 2015). Coronary heart disease (CHD) incidence is higher in type 2 (non-insulin-dependent) diabetes mellitus (Colhoun *et al*., 2004).

Sitagliptin (SIT) is an antidiabetic medication used for the management of type 2 diabetes mellitus (T2DM. the drug inhibits the dipeptidyl peptidase-4 (DPP-4 enzyme). It results in increased insulin secretion which is dependent on glucose from pancreatic cells. Also it decreases the glucose production from liver (Inzucchi *et al*., 2015; Zonoozi *et al*., 2017). Hydroxy methyl glutaryl coenzyme A reductase inhibitor (HMGCo-A), Simvastatin (SIM), at its maximum therapeutic dose that is 80mg/day manifests average decrease in low density lipoprotein cholesterol (LDL-C) along with lowering the level of apolipoprotein B, triglyceride and very low lipoprotein cholesterol and significant raise the level of high density lipoprotein.

Fixed dose combinations (FDCs) are characterized by combining two or more active ingredients as a single pharmaceutical preparation for the administration. FDCs are associated with reduced risk of medication non-adherence, so these combinations are extremely important for chronic disease patients. Keeping in view the above mentioned advantage, care must be practiced as the rationality for the use of FDCs should be based on complete therapeutic principles because concerns have been raised with their irrationality and utility in different countries (Błaszczyk *et al.,* 2018; Auwal *et al.,* 2019; Godman *et al.,* 2020).

Alteration in the optimal dosing of the components in FDCs may occur due to differences in pharmacokinetic profiles and half-lives of different constituents. Increase in frequency of drug-drug interactions may also occur in FDCs. The reasons for these drug-drug interactions might include different profiles of drugs in the combination along with lesser recognition of the differences that may occur in the pharmacogenetic profiles of patients during the development of FDCs. The pharmacokinetic and pharmacodynamics profiles are important in geriatrics because safety profiles may be altered in FDCs (Menditto *et al.,* 2020). There are evidences that inappropriate manufacturing of FDCs are associated with reduced effectiveness and increased toxicity, as well as peak effectiveness also varies at different time intervals. Other concerns with FDCs are high pricing as compared to sum of the individual components. It is difficult to ascertain which component is responsible for the side effect that may arise and sometimes patient receive too little and too much of specific ingredients due to challenges with dose adjustments (Godman *et al.,* 2020).

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A lot of advantages are also associated with FDCs. Improved response rates are observed as compared to monotherapy through different mechanisms of action of the medicines in the combination, the combination of the drugs in the FDC achieves the desired effect more rapidly and FDCs are presumed to reduce the toxicity of one component probably by counteracting the adverse drug reactions of the other (Dubrocq and Rakhmanina, 2018), (Gupta *et al.,* 2018).

US Food and Drug Administration (FDA) has approved SIT and SIM (Ramesh *et al*., 2015), a fixed-dose combination prescription medication. The formulation contains two previously approved SIT and SIM in a single dosage tablet (Ramadan and Kabbara, 2015). Present research was conducted to examine the cytotoxic and genotoxic activity of SIT and SIM alone and in combinations by using Methyl thiazole tetrazolium (MTT) assay and Comet assay on Vero cell line and isolated peripheral blood lymphocytes respectively.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade unless otherwise mentioned were taken from Sigma Aldrich. Histopaque 10771, Ethidium Bromide (EBr), Trizma Base, Disodium EDTA, Normal Melting Agarose (NMA), Low Melting Agarose (LMA), Triton X-100 (Fisher scientific), Phosphate Buffer tablets (Inovatiqa), Dimethyl sulfoxide (Honeywell, analytical pure), Fetal Bovine Serum, heat inactivated and Dulbecco's Modified Eagle Medium (Gibco), Gentamicin 80mg/vial (Pfizer), Streptomycin 1g/vial (Abbot), Penicillin 1 lac unit (Wellona Pharma), MTT dye (bishop).

Equipments

Fluorescence Microscope with power supply and Comet IV software (Perceptive instruments Ltd. England), Horizontal Gel Electrophoresis chamber with Gel Power system GPS-250 (ICCC Meradd), Enzyme Linked Immunosorbent Assay (ELISA) plate reader (Thermo Scientific), Heparinized 3mL BD vacutainers (EDTA k3), 0.22µm MS nylon syringe filters, 0.5 MI Eppendorf tubes (Medical Grade), Falcon centrifuge tubes 15mL, Sterilized flat bottom 96-well plates (Orange Scientific).

Ethics Approval

The Ethics Committee of the Faculty of Pharmacy and Allied Health Sciences, Lahore College for Women University (LCWU), Lahore approved the study of SIT and SIM on isolated peripheral blood lymphocytes and collection of the blood samples at LCWU, Lahore.

Experimental Design and Sample Dose

Two fold dilutions of SIT (31.25, 62.5, 125, 250, 500 and 1000µg/mL), SIM (31.25, 62.5, 125, 250, 500µg/mL), and SIT, SIM combinations (31.25:31.25, 62.5:62.5, 125:125, 250:250, 500:500 and 1000:500µg/mL) were used to evaluate the potential of genotoxicity and cytotoxicity.

Preparation of Cell culture media

Vero cell line (ATCC CRL-1586) was used for cytotoxicity assay, kindly provided by the Quality Operational Laboratory, University of Veterinary and Animal Sciences. Quantification of cell line was carried out by Hemocytometer before starting the MTT assay processing. The measurement of cytotoxicity of SIT and SIM was conducted by the MTT (Methyl Thiazole Tetrazolium) assay, following the basic principle with slight modifications explained by (Sylvester, 2011).

MTT Assay

Media for the cell culture was prepared by dissolving 1.2 gm powdered form of cell culture DMEM in distilled water *q.s* and volume was made upto 100ml along with fetal bovine serum (FBS) and antibiotics. Media for culture was filtered by the help of syringe filter. DMEM supplemented with 20% fetal bovine serum was used to cultivate adherent Vero cell lines. Adherent cells were treated with each test dilution individually and incubated for 72h in 5% CO2, 95% air at 37^{o}C. For each concentration triplet cell wells were used. 20µl MTT dye was added in each well of 96 well plates. The cell plates were incubated for the period of 3h. For the dissolution of formazan crystals, the medium was removed and 100µl dimethyl sulfoxide were added in each well. Absorbance of the culture plates were taken by ELISA plate reader at 570nm (variable between 540 to 720 nm) (Seidl and Zinkernagel, 2013). The purple formazan color produced was directly proportional to the number of viable cells as only viable cells can produce dehydrogenase enzyme to convert yellow tetrazolium MTT dye into purple soluble formazan crystals. So, the % of MTT absorbance was calculated as cell viability and OD of test chemicals alone and in combinations were noted and the Cell Survival Percentage (CSP) calculated using formula (Sharif *et al*., 2017).

$$CSP = \frac{\text{mean OD of test - mean OD of negative control}}{\text{mean OD of Positve control}} \times 100$$

Comet Assay

The assay protocol based upon guidelines of *Singh et al* (Singh *et al*., 1988) was used under alkaline conditions. All the solutions were freshly prepared and the grooved slides were used after repeated washing and cleansing by dipping in methanol for the purpose of removing dust and grease. Basic slides were prepared by dipping 1/3rd of the frosted area of slides into 1% NMA while it is hot, wiped off the lower sides and slides were allowed to dry. Lymphocytes suspension were treated with test dilutions for 2hr at 37^{O}C and then test chemical layers were discarded after micro centrifuge at 3000 rpm for 5 mins and lymphocytes pellet were dissolved in 1mL PBS. 100

µL of 1% LMA was poured over 80µL lymphocytes suspension and layered on the groove of basic slides, covered with cover slips and allowed the gel to harden. The third agarose layer (90µL of 0.5% LMA) added and placed covered slides on flat surface until hardens. The cover slips were removed and slides were allowed to dip completely in lysing solution at 4^0C for 10 hours. Slides were exposed with alkaline buffer solution for 20 minutes to unwind the DNA strands. Slides were placed in the chamber of horizontal gel electrophoresis filled with electrophoresis buffer, avoiding bubbles and slides floating (24 Volts, 300mA, 30min). Followed by neutralization and staining with 80µL of 1X EBr, the slides were immediately evaluated under fluorescence microscope using 400x objective. Comet scoring was done using Comet IV software and comet scoring formula (Comet Assay IV). On each slide, 50 comets were scored (a total of 150 comets for each test dilution) on each slide (Sharif *et al*., 2016).

STATISTICAL ANALYSIS

The results were computed and analyzed by Graph Pad Prism 5 using mean±SD and LSD. Inhibitory concentration (IC50) of the test chemicals alone and in combinations on vero cell line were calculated. Comet IV software developed by Perceptive instruments Ltd. England was used to measure DNA damage extent which determined various parameters of DNA damage.

RESULTS

Cytotoxicity Analysis

The effect of SIT, SIM and their mixture was evaluated o Vero cell line. % cell survival was calculated. The results are presented in fig. 1. All three treatments SIT, SIM and their mixture inhibited proliferation of vero cells except one combination (1000:500). The IC50 of SIT and SIM was 274.3µg/ml and 37.51µg/ml respectively, whereas their combine concentrations were less cytotoxic than SIT alone with an IC50 of 97.70µg/ml. Mean optical density (OD) was used to calculated CSP of treated cells (table 2)

Genotoxicity Assay

The possibility of genotoxicity was investigated using Comet assay with the help of peripheral blood mononuclear cells under non-denaturing condition. Comet tail induction suggests DNA damage. Extent of DNA damage was measured by calculating: Fragmentation Percentage (FP) and Damage index (DI), Tail Length (TL), Tail % Int. (TI %) and Tail Moment (TMom). Results indicated that SIT concentration produced more pronounced genotoxic effect as compared to SIM concentrations. All the attributes fig. 2 exhibited more damage in SIT concentrations. However, the mixture of SIT and SIM produced lesser damage as compared to SIT alone as shown in fig. 3.

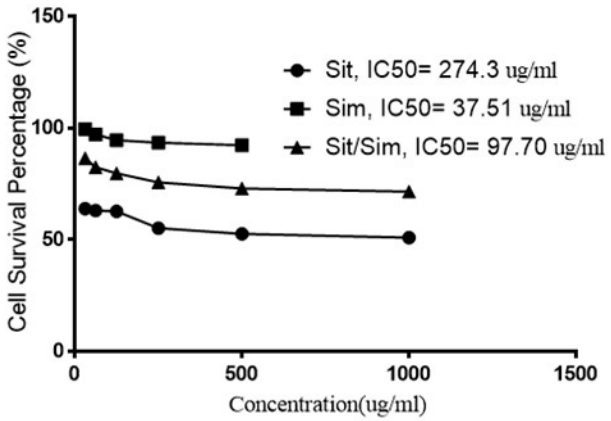

Fig. 1: Cell survival percentage and inhibitory Concentration (IC50) of A. Sitagliptin (SIT) B. Simvastatin (SIM) C. Sitagliptin-Simvastatin against Vero cell lines

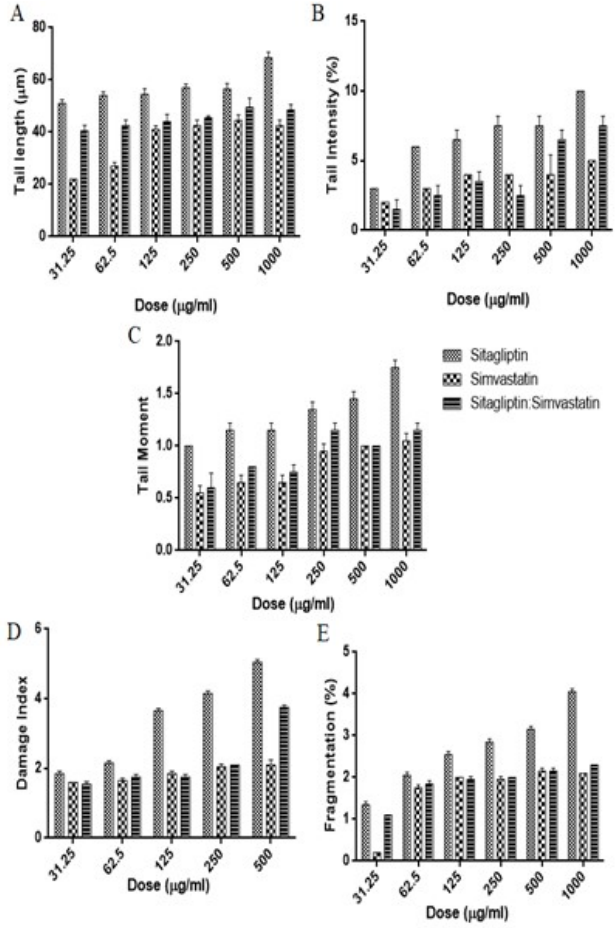

Fig. 2: Effect of Sitagliptin, Simvastatin, Sitagliptin-Simvastatin on A. tail length B. tail intensity C. tail movement D. damage index and E. fragmentation

DISCUSSION

Cytotoxicity and genotoxicity evaluation of antidiabetic and antihyperlipidemic medication requires a special

clinical intervention due to significant increase in patient with type 2 diabetes mellitus along with coronary heart diseases (CHD), demanding long lasting therapeutic regime.

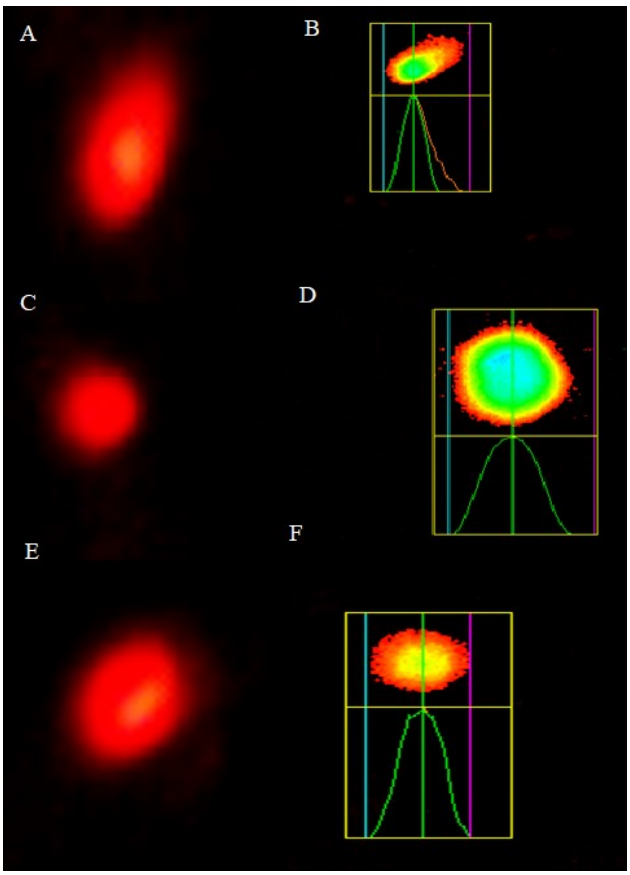

Fig. 3: Comet pictures of lymphocytes treated with A. Sitagliptin C. Simvastatin E. Sitagliptin-Simvastatin. Comet pictures taken by comet software IV treated with B. Sitagliptin D. Simvastatin F. Sitagliptin-Simvastatin

Different dilutions of SIT, SIM and their combination were studied for their genotoxic and cytotoxic effect. The results were compared both for their genotoxic and cytotoxic effects.

Various combinations of SIT and SIM were exposed to Vero cells in this experiment. A synergistic cytotoxic potential was observed and cell viability of Vero cell lines were decreased. As the concentration of SIT (31.25, 62.5, 125, 250 and 500 and 1000µg/ml) raised, the cytotoxic effect of SIT increased and cell survival percentage decreased in dose dependent manner. Previously SIT was reported as cytotoxic agent at higher concentrations (500 and 1000µg/ml) (Gul *et al*., 2013). Similarly, in another study SIT was found to be linked with increased genotoxicity and cytotoxicity in individuals when compared with medical nutrition therapy group (Yuzbasioglu *et al*., 2018). SIM concentrations (31.25, 62.5, 125, 250 and 500µg/ml) were also checked to evaluate the cytotoxic potential. SIM did not showed any signs of cytotoxicity in any tested dilution. (Pedersen and Tobert, 2004). Results corroborated previous findings that SIM does not possess cytotoxic potential. when used at therapeutic and higher concentrations. It supported the clinical use of this anti lipidemic drug in the treatment of coronary heart diseases and cancer patients. (Pedersen and Tobert, 2004). Statins have been reported to possess intrinsic antioxidant activity (Girona *et al*., 1999). Although CSP decreases in combination but interestingly the survival percentage got better when it was compared with SIT treated cells. The increased cell survival percentage in combination as compared to SIT might be attributed to the reported intrinsic antioxidant activity of SIM. SIM (1 and 10mM) was reported to repress vascular endothelial growth factor (VGEF) induced retinal endothelial cells (RECs) proliferation in a concentration-dependent manner, without affecting cell viability (Franzoni *et al*., 2003)

Comet test was performed to determine the Oxidative DNA damage; this assay has been taken as very good technique in human being biomonitoring studies (Azqueta and Collins, 2013). It was observed that all the attributes of comet assay i.e. tail length, tail intensity, tail movement, damage index and fragmentation exhibited an increase at 1000µg/mL SIT concentration. The results confirm the previous investigation where SIT was reported to induced DNA damage at 62.50µg, 250µg, 500 µg and 1000µg dose (Yuzbasioglu *et al*., 2018). SIM however did not exhibited any significant damage at tested concentrations (31.25, 62.5, 125, 250 and 500 µg/mL) in all the attributes of comet assay (fig. 2). Different combinations of SIT and SIM (31.25:31.25, 62.5:62.5, 125:125, 250:250, 500:500 and 1000:500 µg/ml) exhibited a decrease of damage in all comet

Table 1: Optical density and Cell survival percentage CSP of vero cell line

Concentration (µg/ml)	Sitagliptin (OD ± SD) CSP %	Simvastatin (OD ± SD) CSP %	Sitagliptin/Simvastatin (OD ± SD) CSP %
31.25	0.662±0.01, 64	0.95±0.01, 99.6	0.842±0.03, 86.5
62.5	0.645±0.02, 63.1	0.93±0.02, 97.15	0.814±0.05, 82.5
125	0.623±0.03, 62.7	0.91±0.03, 94.6	0.786±0.03, 79.7
250	0.572±0.02, 55.2	0.899±0.02, 93.45	0.753±0.02, 75.75
500	0.56±0.02, 52.7	0.890±0.02, 92.3	0.730±0.01, 72.96
1000	0.549±0.01, 51		0.717±0.02, 71.6

attributes (de Sousa *et al*., 2017). Comet assay revealed that SIM significantly reduced DNA damage. Reduction in DNA tail moment upto the 29.5% was observed in combination. The protective role of SIM might be attributed to the inherent properties of stains where they are involved in the attenuation of doxorubicin-induced increase in p53 along with activation of checkpoint kinase (Chk-1) and stress-activated protein kinase/c-Jun-N-terminal kinase (SAPK/JNK). Statins also provides shielding effect to topoisomerase II against poisons (Damrot *et al*., 2006). Statin treatments were also linked to decrease chromosomal DNA damage and cytotoxicity in patients with dyslipidemia (Donmez-Altuntas *et al*., 2019; Gul *et al*., 2013).

CONCLUSION

It can be concluded that SIT has significant genotoxic effect at some concentrations and also possess cytotoxic activity in dose dependent manner. Augmented cytotoxic and genotoxic response triggered by SIT and SIM combination reduced when tested *in vitro.* However further investigation of this combination is required to confirm and identify the underlying mechanism for the protective role of SIM.

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