

Full Length Research Paper

Inhibition of DPP IV is a suggested mechanism of the immunomodulatory effects of sparfloxacin

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Sparfloxacin is the first marketed amino difluoroquinolone. It is currently marketed in many countries in the world and used widely for the management of urinary tract or respiratory tract infections. Fluoroquinolones could modulate both cellular and humoral immunity. The results of this study showed that Sparfloxacin could significantly suppress the proliferation of both stimulated Balb/c splenocytes and stimulated murine macrophages. Moreover, the release of the IL-6 from these cells was also suppressed. Interestingly, Sparfloxacin inhibits dipeptidyl peptidase IV enzyme (DPP IV) in a dose dependent manner with an IC₅₀ of 29.6 μM. These findings suggest that DPP IV inhibition by Sparfloxacin could be one of the mechanisms by which Sparfloxacin exerts its immune-modulatory activities.

Key words: Sparfloxacin, fluoroquinolones, dipeptidyl peptidase, DPP IV, immunomodulatory, IL-6, mitogen, raw cells.

INTRODUCTION

Quinolones are widely used antibacterial agents. The development of these drugs started with the non-fluorinated drug nalidixic acid. Quinolones and their new fluoroquinolone derivatives offer a broader spectrum of activity than older agents and increased potency against bacteria resistant to older agents (Fuhr et al., 1993).

The fluoroquinolones (FQs) are most often used today for the treatment of bacterial infections, such as urinary tract infections or respiratory tract infections.

Sparfloxacin is the first marketed amino difluoroquinolone and is currently marketed in the United States. The fluorine atom at position C-8 (Figure 1) is thought to increase sparfloxacin's absorption and plasma half-life, and to increase its activity against gram positive species *Streptococcus pneumoniae* and *Staphylococci* (Schentag, 2000). Quinolones, in general interfere with

bacterial DNA synthesis by blocking topoisomeras (DNA gyrase) activities, however, differences in the structure among quinolones specify their exact target of their antibacterial activity. Sparfloxacin inhibits primarily Topoisomerase II of *S. pneumoniae* while topoisomerase IV is considered its secondary site of action (Nakanishi et al., 1991; Okuda et al., 1991; Schentag, 2000; Tanaka et al., 1991).

FQs are thought out relatively safe and well-tolerated drugs (Albertson et al., 2010; Stahlmann, 2002). However, FQs were found to affect both cellular and humoral immunity (Dalhoff, 2005). Some FQs, like ciprofloxacin, were found to inhibit in vitro as well as in vivo the synthesis of TNF-α, IL-1, IL-6, and IL-12. The kinetics of the inhibitory effect on TNF-α production triggered by ciprofloxacin occurred at a very early step of TNF-α synthesis (Lahat et al., 2007; Reato et al., 2004). The basic mechanisms underlying FQs immunomodulatory activity have not been elucidated in a comprehensive and satisfying manner. The precise cascade of intracellular processes leading to stimulatory

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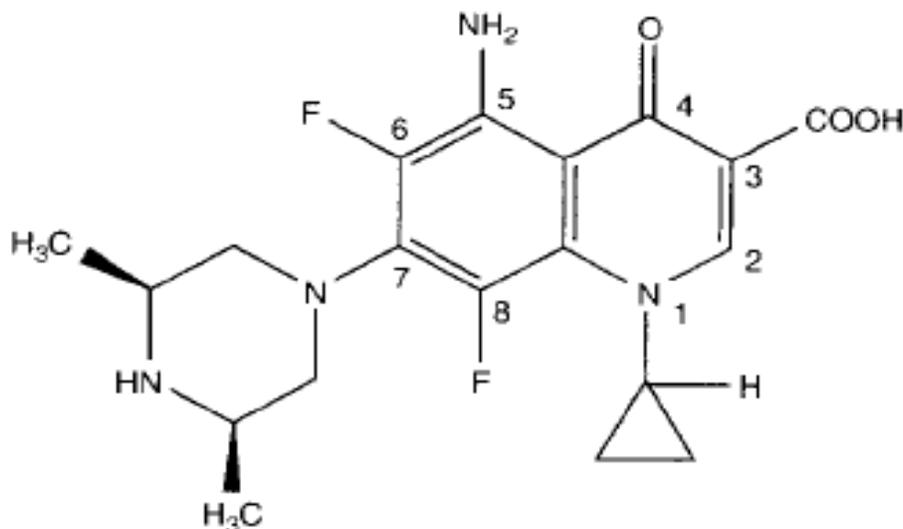


Figure 1. Molecular structure of Sparfloxacin.

or inhibitory effects on cytokines, chemokines and other components of the immune system needs to be elucidated.

Several reports, however, have tried to explain the various immunomodulating effects of FQs in eukaryotic cells, this includes the effects of FQs on intracellular cyclic AMP (cAMP) and phosphodiesterases, the effects of certain members of this group on critical transcription factors such as NF-kappaB, AP-1, NF-IL-6 (Galley et al., 2000; Ogino et al., 2009; Wada et al., 2008). Recent reports have demonstrated the inhibitory potential of Gemfloxacin, FQ member, on two enzymes Glycogen Synthase Kinase-3 β (GSK-3 β) (Taha et al., 2008) and Dipeptidyl peptidase IV (DPP IV) Al-masri, 2008 #12). The inhibition of these targets could justify, at least partly, the reported immunomodulatory effects of this drug.

Dipeptidyl peptidase IV (DPP IV, CD26), is a multifunctional type II transmembrane glycoprotein. It is expressed both on the cell surface of various immune cell type; leukocyte subsets such as T, B, and natural killer lymphocytes and macrophages, and nonimmune cell types such as epithelial cells, several types of endothelial cells and fibroblasts, also a soluble form of CD26, lacking the cytoplasmic tail and transmembrane region, is found in plasma and other biological fluids (Bjelke et al., 2006). DPP IV is a Ser peptidase that has an extracellular domain with DPP IV enzymatic activity and a short cytoplasmic domain. It interacts with extracellular molecules and is also involved in intracellular signal transduction cascades (Thompson et al., 2007).

The unique substrate specificity of DPP IV results in playing a key role in the catabolism of a number of chemokines and cytokines, neuropeptides, immunopeptides, and peptide hormones, containing the X-pro or X-Ala amino terminal sequences such as glucose-dependent insulinotropic polypeptide (GIP) and

glucagon-like peptide-1 (GLP-1) (Reinhold et al., 2006). This makes DPP IV to have a variety of roles in metabolism, immunity, endocrinology and cancer biology. DPP IV is a new and successful type 2 diabetes therapeutic target (Gerich, 2010; Yu et al., 2010). Many recent reports have demonstrated that DPP IV serves as an important regulator of immune responses by affecting T cell activation, proliferation, and cytokine production (Ansorge et al., 2009; Gerich, 2010; Reinhold et al., 1994; Reinhold et al., 2009; Taha et al., 2008; Yu et al., 2010).

The current study commenced by the investigation of the effect of Sparfloxacin on both cellular proliferation and IL6 production in mitogenic stimulated splenocytes and stimulated macrophage cell line (RAW). Furthermore, the *in vitro* inhibition of splenocyte DPP IV using escalating concentrations of Sparfloxacin was investigated in order to reach preliminary conclusions about the underlying mechanism of Sparfloxacin immunomodulatory effect.

MATERIALS AND METHODS

The following materials were obtained from their sources and used without any purification: Culture media (RPMI-1640, Lonza, Euro-clone), Eagle's modified essential medium (EMEM, Lonza), Fetal Bovine Serum (Gibco), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (Lonza), L-Glutamine (Lonza, Euro-clone), Penicillin-Streptomycin (Lonza), Gentamicin (Lonza), 2-Mercaptoethanol (Sigma), Phosphate Buffer Saline (Lonza, Gibco), Trypsin-EDTA (Lonza, Euro-clone), Trypane Blue Stain (Sigma), Lipopolysaccharides (Sigma), 3(4, 5 diamethyl 1-2-thiazoly) 2,5-diphenyl-2H-tetrazolium (MTT) (Sigma), 3(4,5 diamethyl 1-2- thiazoly) 2,5-diphenyl-2H-tetrazolium (MTT) non-radioactive cell proliferation kit (Promega), ELISA kit human IL-6 (Peprotech), Tween-20 (Sigma), Bovine Serum Albumin (BSA) (Sigma), 2, 2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABSA), Ammonium Chloride (Sigma), Concanavalin A (Sigma), Mitomycin C (Sigma), Sparfloxacin (Sigma), Macrophages

stimulating factor (Sigma).

Mice

Specific pathogen-free inbred female Balb/c mice (6 to 8 weeks old, 19 to 21 g weight) were housed in polystyrene cages in an air controlled room. All animals were maintained at laboratory diet and tap water ad libitum. Animals were acclimatized for 1 week in the lab prior to usage. All animal experiments comply with the guide for the care and use of laboratory animals published by the US national institutes of health.

Single cell suspension of splenocytes

Splenocytes suspension was prepared as described previously (Fararjeh et al., 2008; Mosmann, 1983). Briefly, the spleens of Balb/c mice were used to prepare the splenocytes suspension (2.0×10^6 cells/ml).

To disrupt mononuclear cells out of spleens, each spleen was teased between the frosted edges of two sterile microscopic slides, in a sterile tissue culture dish containing 10 ml complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine). The released cells were collected from the tissue plates and were transferred to a sterile centrifuge tube. The tubes were centrifuged at 1200 rpm for 7 min at 4°C in a swing bucket refrigerated centrifuge (Hittech, Germany). Supernatant was discarded, and the pellet was resuspended in 3 ml of RBC lysing buffer containing 0.83% NH₄Cl in 100 mM Tris buffer, pH 7.4 (Sigma) and kept at room temperature for 3 min. The lysed RBCs were washed out 3 times in 10 ml of RPMI-1640 medium by centrifugation at 1200 rpm for 3 min using refrigerated.

Mitogen splenocytes proliferation assay

Cell count and viability were assessed by trypan blue dye exclusion. In mitogen proliferative assays, splenocytes were incubated with 5 µg/ml concanavalin A (Con A) (Reinhold et al., 1997b), then MTT assay was performed.

Proliferation assay of RAW 264.7 murine macrophage cell line

The RAW 264.7 murine macrophage cell line were grown at 37°C in RPMI-1640 medium supplemented with 10% FBS, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) Buffer (10mM), L-glutamine (2 mM), Gentamicin (50 µg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 mg/ml) in a humidified 5% CO₂ atmosphere. Cells were incubated with Sparfloxacin at various concentrations and stimulated with LPS 100 ng/ml for various times.

MTT assay for cell viability

The RAW 264.7 were plated at a density of 2.2×10^6 cells/ml into a 96 well plate, containing 90 µl of RPMI-1640 medium supplemented with 10% FBS. The cells were incubated 1 h with Sparfloxacin at various concentrations by adding 100 µl of each (200, 100, 50, 25, 12.5 and 6.25) µg/ml, then stimulated with LPS by adding 10 µl of 2 µg/ml LPS solution. Following 18 h of incubation after seeding, 20 µl of PBS containing 5 mg/ml MTT was added. After incubation for 2.5 h at 37°C the medium was discarded and the Formazan blue (that had formed in the cells) was dissolved in 200 µl DMSO.

The optical density at 570 nm was determined with a microplate reader, with that of the formazan formed in the control (untreated) cells taken as 100% viability (Fararjeh et al., 2008; Mosmann, 1983).

Measurement of cytokine (IL-6) expression

The effect of Sparfloxacin at various concentrations on Cytokine (IL-6) expression of splenocytes or RAW cell line was determined by ELISA kit as described in the manufacturer's instructions (Peprotech). All experiments were performed in triplicate. The stimulated cells were incubated with various concentrations of Sparfloxacin. Following 18 h of incubation after seeding the inflammatory cytokine concentration of each culture supernatant was measured (Reinhold et al., 1997b).

Quantification of DPP IV activity by a spectrophotometric assay

The DPP IV activity was quantified by a colorimetric assay that measures the release of paranitroaniline (pNA) as previously described (Al-masri et al., 2009; Reinhold et al., 1997b), with minor modification. Briefly, sparfloxacin was dissolved in DMSO and diluted with Tris buffer (pH 7.5) for subsequent enzymatic assay. The assay was conducted using DPP IV of splenocytes, the assay is based on the cleavage of chromogenic substrate (H-Gly-Propara-Nitroaniline) by DPP IV to release para-nitroaniline (pNA) measured at 405 nm. Briefly, 200 µL splenocytes cell suspension was mixed with the specified quintiles of previously prepared Sapr solution. Thereafter, the final volume of the reaction mixture was completed by Tris buffer (pH 7.5, 50 mm) to 980 µL and incubated at 37°C for 30 min. Subsequently, 20 µL of 0.3 M of the substrate was added to the reaction mixture and incubated at 37°C for at least 1 h. Thereafter reaction mixture was centrifuged (2000 rpm) and the clear supernatant was obtained the absorbance was measured at 405 nm by a UV spectrophotometer against a blank that contain the same mixture but without enzyme. The obtained absorbances were compared to a negative control (enzymatic solution without inhibition). A standard DPP IV inhibitor (P32/98 from Biomol, Germany) was employed as positive control.

Inhibition of DPP IV by the test material was calculated from the residual activity of the uninhibited DPP IV control using the following formula:

The percent inhibition was plotted against the logarithmic transformation of the corresponding test extract concentrations for determining the IC₅₀ values (concentration required to give 50% inhibition (IC₅₀). All assays were triplicated and the calculated inhibition percentages were the mean of 3 observations.

RESULTS

Proliferation assay

The effect of Sparfloxacin on Con A stimulated splenocytes and on LPS- stimulated RAW macrophages was determined by incubation of viable cells for 18 h in the presence and absence of various concentrations of Sparfloxacin. As shown in Figures 2 and 3, the proliferation of both types of cells was strongly suppressed at Sparfloxacin concentrations above 12.5 µM. It was found that 85% inhibition on Con A

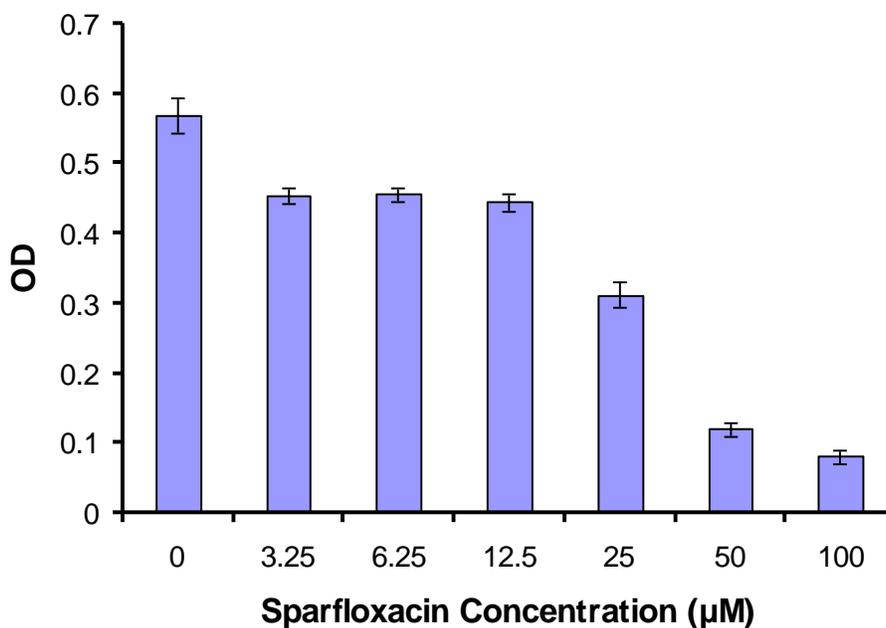


Figure 2. Proliferation assay of Balb/c mice splenocytes (stimulated with Con A) after incubation with various concentrations of sparfloxacin.

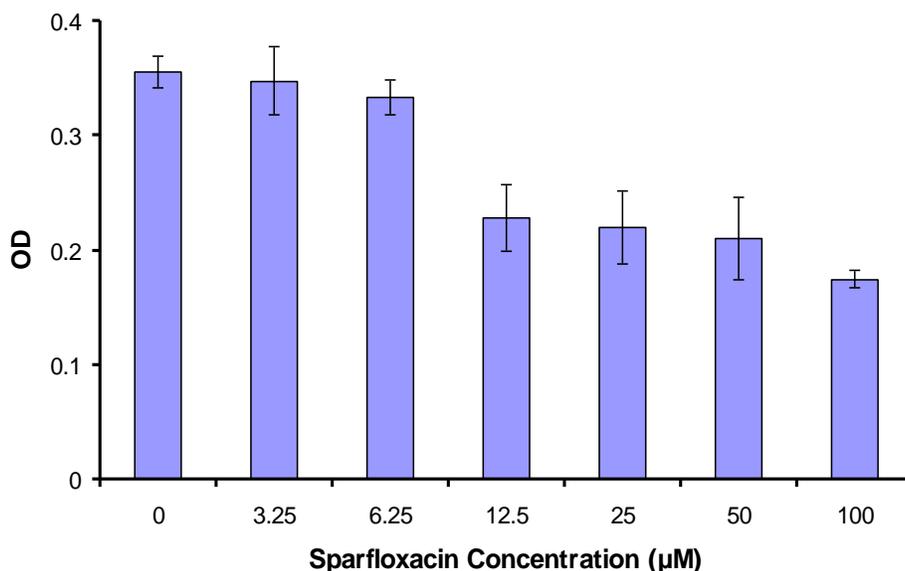


Figure 3. Proliferation assay of RAW 264.7 murine macrophage cell line (stimulated with LPS) after incubation with various concentrations of sparfloxacin.

stimulated splenocytes and 50% inhibition on LPS stimulated murine RAW cell-line are achieved at a Sparfloxacin concentration of 100 µM.

Sparfloxacin effect on 1L-6 production

To answer the question whether the suppressive effect

of Sparfloxacin on proliferation of mitogen- stimulated splenocytes and murine macrophages correlates with a decrease in production of different cytoki-nes, we measured the concentrations of IL-6 in supernatants of Con A stimulated splenocytes and LPS stimulated Raw macrophages in the presence or absence of various concentrations of Sparfloxacin. The level of IL 6 cytokine was quantified after 18 h incubation by enzyme

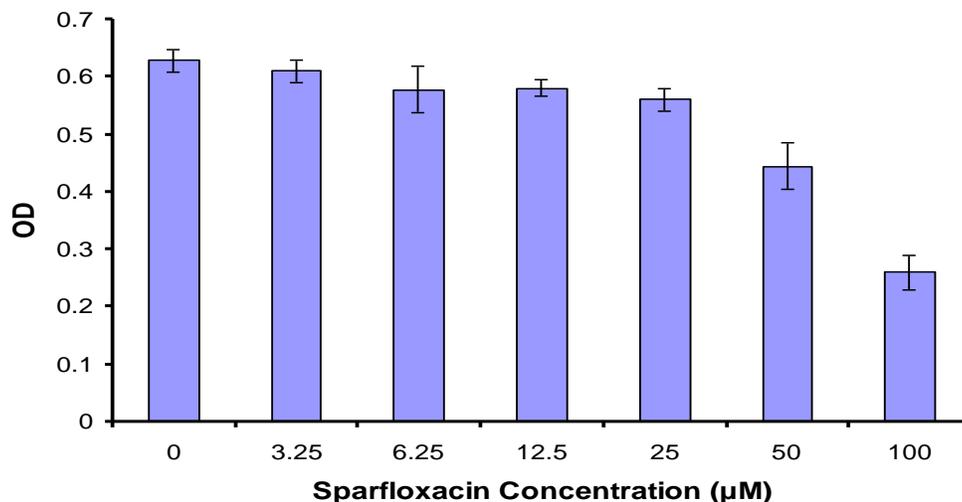


Figure 4. IL-6 production in Balb/c mice splenocytes (stimulated with Con A) after incubation with various concentrations of Sparfloxacin.

immunoassays methods. As shown in Figures 4 and 5, Sparfloxacin has significantly suppressed the release of IL-6 in a dose dependent manner in both the stimulated splenocytes and stimulated murine macrophage cell line.

DPP IV inhibition

The inhibitory action of Sparfloxacin was experimentally validated against DPP IV of freshly isolated splenocytes incubated for a period of 30 min with escalating concentrations of Sparfloxacin using the H-Gly-Pro-pNA as substrate. The enzymatic reaction progression was monitored through the release of para-nitroaniline. The *in vitro* activity was expressed as the concentration of Sparfloxacin that inhibited enzyme activity by 50% (IC₅₀). Different Sparfloxacin concentrations were capable of suppressing significantly the DPP IV enzymatic activity with an estimated IC₅₀ of 29.6 µM (Figure 6).

DISCUSSION

During the last years, posttranslational modification by proteolysis has been recognized as one of the mechanisms involved in regulating the biological activities of many chemokines. As suggested by recently published findings, the protease dipeptidyl peptidase IV (DPP IV), a highly specific and unique aminopeptidase, may be of special importance in modulating chemokine activity. Targeting DPP IV provided a potent therapeutic approach for the treatment of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (MS), and it represents a novel and efficient therapy for autoimmune disease of the central nervous system (Reinhold et al., 2006). Many reports concluded that

quinolones exert their effects on the synthesis of various cytokines and chemokines through modulation of key cellular transcription factors (Dalhoff, 2005).

Recent reports have demonstrated the inhibitory potential of Gemfloxacin, FQ member, on DPP IV. The inhibition of these targets could justify, at least partly, the reported immunomodulatory effects of this drug (Taha et al., 2008). Moreover, many synthetic competitive DPP IV inhibitors significantly suppress production of IL-2, IL-1 and IFN-γ in PWM-stimulated T cells. Under the same conditions, the inhibitors are capable of inducing the secretion of the latent form of the 'immunoinhibitory' cytokine transforming growth factor-β₁, (TGF-β₁) (Reinhold et al., 1997a; Reinhold et al., 1997b). It has been shown, however, that high levels of IL-6 could induce the release of TGF-β₁ (Zhou et al., 1991). IL-6 is a multi-functional protein with roles in host defense, acute phase reactions, inflammation and immunity. It can be regulated by a number of transcription factors, apparently depending upon the cell type and stimulus (Zhou et al., 1991).

In this study, it has been demonstrated that sparfloxacin has significantly suppressed the cellular proliferation of both Con A stimulated splenocytes that are freshly prepared from Balb/c mice and LPS stimulated RAW murine macrophage cell line. The inhibitory effect of Sparfloxacin was more pronounced on splenocytes proliferation compared with its effect on RAW cell line (Figures 2 and 3).

To answer the question whether the suppressive effect of sparfloxacin on proliferation of mitogen stimulated splenocytes and murine macrophages correlates with a decrease in production of different cytokines, we measured the concentrations of IL-6 in supernatants of stimulated splenocytes and stimulated RAW macrophages in the presence or absence of various

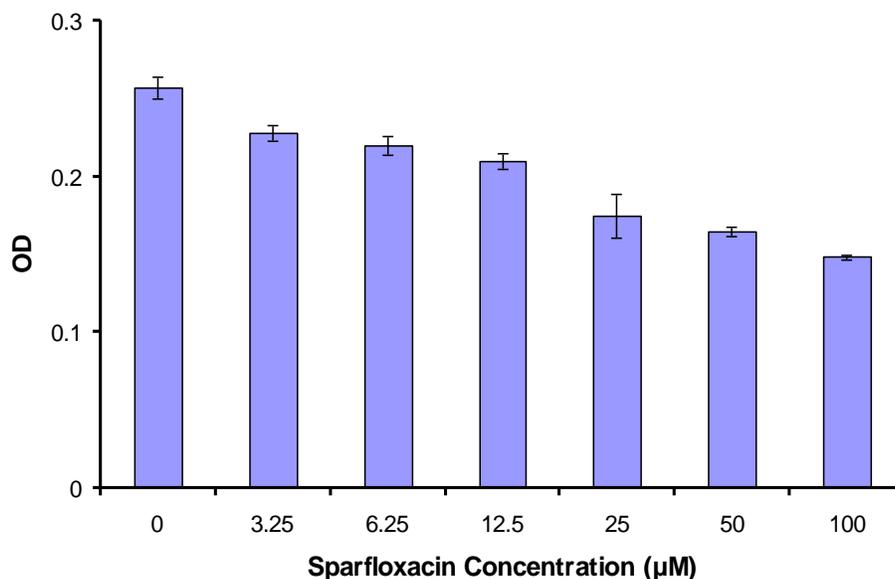


Figure 5. IL-6 production in RAW 264.7 murine macrophage cell line (stimulated with LPS) after incubation with various concentrations of Sparfloxacin.

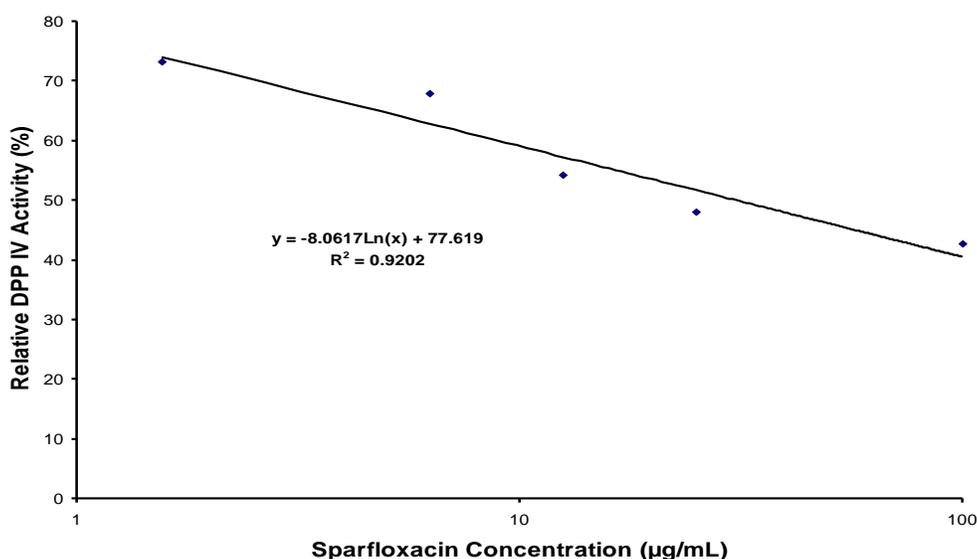


Figure 6. The effect of variable Sparfloxacin concentrations on the relative activity of DPP IV. Data are expressed as means of three replicates.

concentrations of Sparfloxacin. As shown in Figures 4 and 5, Sparfloxacin has significantly suppressed the release of IL-6 in a dose dependent manner in both the stimulated splenocytes and stimulated murine macrophages. However, the degree of inhibition was not similar in both cell systems. As shown in Figures 4 and 5, the production of IL-6 was significantly lower in the case of splenocytes at 100 µM of Sparfloxacin. IL-6 has been shown to be regulated by a number of biological factors, including DPP IV. Many studies have demonstrated suppressive

activities of the DPP IV inhibitors on the release of IL 6 from different immune cells (Reinhold et al., 1997a; Reinhold et al., 1997b).

To investigate the effect of Sparfloxacin on the DPP IV activities, escalating concentrations of Sparfloxacin were incubated with splenocytes in the presence of the DPP IV substrate. The release of para-nitroaniline was monitored in the presence and absence of Sparfloxacin. Interestingly, Sparfloxacin inhibits DPP IV activities with an IC₅₀ of 29.6 µM (Figure 6).

DPP IV inhibitors, generally, suppress antigen induced proliferation and IL-6 production in immune cell lines. Here, in our study, we demonstrate that sparfloxacin which showed an effective DPP IV inhibition, could suppress cellular proliferation and IL-6 production in both Con A stimulated splenocytes and LPS-Raw macrophages. This sparfloxacin immunomodulatory effect could be attributed, at least in part, to DPP IV inhibitory activities.

Intriguingly, a major noncardiac side effect of sparfloxacin is the pronounced hypoglycemia which could also be a side effect of most FQs. This side effect can be also correlated to its DPP IV inhibitory potential. Incretins: glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are well known DPP IV substrates. Incretins stimulates insulin biosynthesis and secretion and reduces glucagons release. These peptides (incretins) have very short half-lives because of their rapid degradation by DPP IV (approximately two minutes) (Adelhorst et al., 1994; Larsen et al., 2004; Rolin et al., 2004). Therefore, inhibiting DPP IV should promote the hypoglycemic effects of GLP-1 and GIP. Therefore, the reported hypoglycemic effect of Spar and other fluoroquinolones could be attributed at least partly to DPP IV inhibition.

Conclusions

In conclusion, the findings of the present in vitro and ex vivo studies demonstrate that DPP IV inhibition by sparfloxacin could be one of the mechanisms by which Sparfloxacin exerts its immuno modulatory activities. This was demonstrated, in this study, on both stimulated splenocytes and murine macrophages. These results may have also important implications for the treatment of human diseases with a putative autoimmune pathogenesis. Sparfloxacin could act as a potentially powerful and safe lead for the development of a novel class of anti inflammatory agents. Further work is still necessary to enhance its efficacies via optimizing their structure activity relationship (SAR).

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