Chapter 2: Hypothesis and Objectives

2.1 HYPOTHESIS

The hypotheses to be tested in this study are that moxifloxacin

1) possesses anti-inflammatory properties in addition to its conventional antimicrobial activity, and
2) neutralizes the pro-inflammatory activities of the pneumococcal pore-forming toxin, pneumolysin.

2.2 GENERAL OBJECTIVES

This study has been designed to investigate the *in vitro* immunomodulatory effects of moxifloxacin, on stimulated and unstimulated human blood neutrophils and T lymphocytes.

2.2.1 SPECIFIC OBJECTIVES

- To evaluate the effects of moxifloxacin on oxidant production by PMA and fMLP-activated neutrophils (using luminol- and lucigenin-dependent chemiluminescence), and the release of elastase (colorimetric procedure) by fMLP/CB-activated neutrophils.

- To measure store-operated Ca^{2+} influx into fMLP-stimulated neutrophils using a radiometric procedure.

- To evaluate the potential of moxifloxacin to antagonize the injurious and pro-inflammatory interactions of the pore-forming cytotoxic and pro-inflammatory microbial toxin, pneumolysin, with neutrophils.

- To investigate the effect of moxifloxacin on mitogen (phytohaemagglutinin; PHA)-activated proliferative responses of human mononuclear leukocytes (MNL) using a conventional assay based on the uptake of radiolabelled thymidine.

- Flow cytometric assessment of the effects of moxifloxacin on the expression of CD25 (interleukin-2 receptor) on MNL activated with PHA.

- Measurement of the cytokine profiles (both pro-inflammatory and anti-inflammatory cytokines) of PHA-activated MNL using suspension bead protein array technology (Bio-Plex™).

Chapter 3: Effects of Moxifloxacin on Human Neutrophil Functions

3.1 INTRODUCTION

Neutrophils are an essential component of the inflammatory response and for the resolution of microbial infection. On encountering microorganisms, neutrophils engulf these microbes into a phagosome, which fuses with intracellular granules to form a phagolysosome, in which the organisms are killed after exposure to antimicrobial enzymes and peptides, and reactive oxygen species (ROS). The laboratory research described in this chapter was undertaken with the primary objective of determining the effects of moxifloxacin on human neutrophil activities, with emphasis on superoxide production, elastase release and calcium fluxes.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Moxifloxacin was kindly supplied by Bayer Healthcare AG, Leverkusen, Germany. Moxifloxacin was dissolved in sterile distilled water and used in the assays described below at final concentrations of 0.5 – 20 µg/ml. Recombinant pneumolysin was kindly supplied by Professor T.J. Mitchell, Division of Infection and Immunity, University of Glasgow, UK. Unless indicated all other reagents were purchased from Sigma Diagnostics (St. Louis, Mo, USA).

3.2.2 Neutrophil isolation

Purified human neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult volunteers and separated from mononuclear leukocytes by centrifugation on Histopaque®-1077 cushions at 400 g for 25 minutes at room temperature. The granulocyte/erythrocyte fractions were sedimented with 3% gelatin for 15 minutes at 37 ºC to remove most of the red blood cells (RBC). After centrifugation, residual RBCs were removed by selective lysis with 0.84% ammonium chloride at 4 ºC for 10 minutes. The neutrophils, which were routinely of high purity and viability (>90%), were resuspended to 1 x 10^7 cells/ml in phosphate-buffered saline (PBS; 0.15M, pH 7.4) and held on ice until use. Purity of isolated neutrophils was assessed microscopically and assessment of viability was done by dye-exclusion using 0.1% methylene blue.

3.2.3 Oxidant generation

The generation of superoxide and oxidants derived from the MPO/H_2O_2/halide system, were measured using lucigenin (bis-N-methylacridinium nitrate) and luminol (5-amino-2, 5-dihydro-1, 4-phthalazinedione)-enhanced chemiluminescence (LECL) methods respectively (Minkenberg and Ferber, 1984). Neutrophils (1 x 10^6 final) were preincubated for 15 minutes in 900 µl indicator-free Hanks balanced salt solution (HBSS, pH 7.4, Highveld Biological, Johannesburg, RSA) in combination with 0.2 mM lucigenin or 0.1 mM luminol in the presence and absence of moxifloxacin (1-20 µg/ml, final), prior to activation with phorbol 12-myristate 13-acetate (PMA, 25 ng/ml final). Spontaneous and PMA-activated LECL responses were recorded using an LKB Wallac 1251 chemiluminometer (Turku, Finland) and the readings were recorded as mV/sec. Additional experiments were performed, in the same manner, to investigate the effect of moxifloxacin on the LECL response of neutrophils activated with the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1 µM, final) using a Lumac Biocounter® M2010 (Lumac Systems, Schaumberg, The Netherlands). LECL readings were integrated for 10-second intervals and recorded as relative light units (r.l.u).

3.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils (1 x 10^6 cells/ml final) suspended in HBSS in the presence and absence of moxifloxacin (1, 2.5, 5, 10 and 20 µg/ml) was incubated for 10 minutes at 37 ºC. The stimulant fMLP (1 µM final) in combination with cytochalasin B (CB, 1µM) was then added to the cells, which were incubated for 15 min at 37˚C, after which the tubes were transferred to an ice bath, followed by centrifugation at 250 g for 10 minutes to pellet the cells. The neutrophil-free supernatants were assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.,* 1982). Briefly, 125 µl of supernatant fluid (SNF) was added to 125 µl of the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide, 3 mM in 0.05 M Tris-HCl (pH 8.0). Elastase activity was monitored at the wavelength of 405 nm using a Power $Wave_x$ plate spectrophotometer (Bio-Tec instruments, Inc.,) and the results expressed as the mean

percentages of the total cellular enzyme content released during activation by the corresponding fMLP/CB-activated, drug-free control systems.

3.2.5 Radiometric assessment of Ca^{2+} fluxes

Calcium-45 chloride ($^{45}Ca^{2+}$, specific activity 18.53 mCi/mg, Du Pont NEN Research Products, Boston, MA, USA) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} influx in resting and moxifloxacin-treated neutrophils, as well as efflux of the cation. In the assays of Ca^{2+} influx and efflux described below, the radiolabelled cation was used at a fixed, final concentration of 2 µCi/ml, containing 50 µM cold carrier Ca^{2+} (as $CaCl_2$) and the final assay volumes were 5 ml containing a total of 1 x 10^7 neutrophils. The standardization of the procedure used to load the cells with $^{45}Ca^{2+}$ has been described previously (Anderson and Goolam Mahomed, 1997).

3.2.5.1 Influx of $^{45}Ca^{2+}$ into moxifloxacin-treated neutrophils

This procedure was used to measure the magnitude of Ca^{2+} influx following activation of neutrophils with the chemotactic tripeptide fMLP, as well as the effect of moxifloxacin on Ca^{2+} influx. To measure the net influx of $^{45}Ca^{2+}$ into fMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were preincubated for 15 min at 37 ºC in Ca^{2+}-replete HBSS, then pelleted by centrifugation and resuspended to 1 x 10^7/ml in HBSS containing 250 µM cold Ca^{2+}. Pre-loading of neutrophils with cold Ca^{2+} was undertaken to ensure that intracellular Ca^{2+} stores were replete, thereby minimizing spontaneous uptake of $^{45}Ca^{2+}$ in the influx assay. The Ca^{2+}-loaded neutrophils (2 x 10^6/ml) were then preincubated for 10 min at 37 ºC in HBSS containing a final concentration of 50 µM cold, carrier Ca^{2+} in the presence and absence of moxifloxacin (10 µg/ml final). This was followed by the simultaneous addition of fMLP (1 µM) and $^{45}Ca^{2+}$ (2 µCi/ml), or $^{45}Ca^{2+}$ only to control, unstimulated systems. The influx of $^{45}Ca^{2+}$ into fMLP-activated neutrophils was determined 5 min later when influx is complete, and the values compared with the uptake of the radiolabelled cation by identically processed unstimulated cells using liquid scintillation spectrometry. Briefly, the cells were washed twice in ice-cold HBSS, followed by lysis of the cell pellets with 0.5 ml of Triton X-100/ NaOH

(0.1%: 0.05 M), addition of scintillation cocktail and detection of the amount of cell-associated radioactivity (counts per minute) using a Tri-Carb – 2100TR (Packard) liquid scintillation spectrometer. Additional experiments were performed in the same manner to measure the influx of $^{45}Ca^{2+}$ into pneumolysin (8.37 ng/ml)-activated neutrophils.

3.2.5.2 Efflux of $^{45}Ca^{2+}$ from moxifloxacin-treated neutrophils

To measure net efflux of $^{45}Ca^{2+}$ from neutrophils, uncomplicated by concomitant influx of the radiolabelled cation, the cells (10^7/ml) were loaded with $^{45}Ca^{2+}$ (2 µCi/ml) for 15 min at 37 ºC in HBSS. The neutrophils were then pelleted by centrifugation, washed once with, and resuspended in Ca^{2+}-replete HBSS. The $^{45}Ca^{2+}$-loaded neutrophils (2 x 10^6/ml) were then pre-incubated for 10 min at 37 ºC followed by addition of moxifloxacin (10 µg/ml final), activation with fMLP (1 µM) and measurement of the net efflux of $^{45}Ca^{2+}$ after a fixed time period of 60 sec. Reactions were stopped by adding 10 ml ice-cold Ca^{2+}-replete HBSS to the tubes, which were then transferred immediately to an ice-bath. The cells were pelleted by centrifugation at 400 g for 5 min followed by washing with ice-cold Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of Triton X-100/ NaOH (0.1%: 0.05 M), and radioactivity assayed in a scintillation spectrometer.

3.2.6 Cellular ATP levels

Measurement of cellular ATP levels was performed to investigate the cytotoxic potential of moxifloxacin for neutrophils. Neutrophils (1 x 10^6 cells/ml) were incubated in the presence and absence of moxifloxacin (2.5, 5, 10 and 20 µg/ml) for 10 min in a 37ºC waterbath. Following incubation, 20 µl of cell suspension were added into pre-prepared chemiluminometer cuvettes containing 100 µl of nucleotide releasing agent (NRS), which causes release of ATP from the cells, and 30 µl of ATP assay mix dilution buffer (FL-AAM). After vortexing, 20 µl of ATP assay mix was added to the mixture, and chemiluminescence measured using the Lumac Biocounter® 2010M and the results (r.l.u.) converted to nmoles/10^6 cells using a standard curve.

3.2.7 STATISTICAL ANALYSIS

The results of each series of experiments are expressed as the mean values ± the standard error of the mean (SEM). Levels of statistical significance were calculated by paired Student's *t*-test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. P values of ≤ 0.05 were considered significant.

3.3 RESULTS

3.3.1 Effects of moxifloxacin on luminol- and lucigenin-enhanced chemiluminescence responses of neutrophils activated with fMLP or PMA

Neutrophils (1 x 10^6 cells/ml) were pre-incubated in the presence of 0.1mM luminol (5-amino-2,5-dihydro-1,4-phthalazinedione) or 0.2 mM lucigenin (10-10'-dimethyl-bis-9,9'-biacridinium nitrate) with moxifloxacin at final concentrations of 1, 2.5, 5, 10 and 20 µg/ml for 15 minutes at 37 °C. The cells were then exposed to the activators of oxidant production, fMLP (1 µM) or PMA (25 ng/ml). The results of these experiments with fMLP and PMA are shown in Tables 2.1a, b and 2.2a, b respectively. In both luminol- and lucigenin-chemiluminescence systems, moxifloxacin did not affect oxidant (superoxide and oxidants derived from the MPO/H_2O_2/halide system) generation by either fMLP- or PMA-stimulated neutrophils.

3.3.2 Effect of moxifloxacin on elastase release by fMLP/CB-activated neutrophils

Moxifloxacin at concentrations of 1, 2.5, and 5 µg/ml did not affect the release of elastase by neutrophils measured 30 min after the addition of fMLP/CB, while at concentrations of 10 and 20 µg/ml release of elastase was significantly increased. These results are presented in Figure 2.1. Moxifloxacin alone did not affect the release of elastase from neutrophils. The values for the untreated control were 100 ± 9, and for moxifloxacin (1, 2.5, 5, 10 and 20 µg/ml)-treated cells were 112 ± 9, 110 ± 6, 122 ± 3, 117 ± 3 and 110 ± 6 percentage of control/10^6 cells respectively.

3.3.3 Effect of moxifloxacin on neutrophil ATP levels as an index of viability

The ATP content of the neutrophils was measured using the luciferin-luciferase firefly luminescence method (Jabs *et al.,* 1997). These experiments were performed to investigate the cytotoxic potential of moxifloxacin for neutrophils. Neutrophils were exposed to moxifloxacin (2.5 - 20 µg/ml) for 10 min at 37 °C. The values for untreated cells were 65.2 ± 5.2 nmoles/10^6 cells while the values for those treated with moxifloxacin (2.5, 5, 10 and 20 µg/ml) were 64.4 ± 5.6, 67.4 ± 7.9, 70.8 ± 5.7, and 64.9 ± 5.7 nmoles/10^6 cells respectively. These results indicate that treatment of neutrophils with moxifloxacin at concentrations of up to 20 µg/ml does not affect cell viability.

3.3.4 Effects of moxifloxacin on $^{45}Ca^{2+}$ fluxes

3.3.4.1 Effects on influx of $^{45}Ca^{2+}$

For these experiments, neutrophils were preloaded with cold Ca^{2+} (to minimize the spontaneous uptake of $^{45}Ca^{2+}$ in the influx assay), transferred to Ca^{2+}-free HBSS, and incubated with moxifloxacin for 10 min at 37 °C prior to the simultaneous addition of fMLP (1 µM) and $^{45}Ca^{2+}$ (2 µCi/ml). Activation of control, drug-free neutrophils with fMLP resulted in a delayed influx of $^{45}Ca^{2+}$, which occurred after a lag phase of 30 – 60 sec. The influx of $^{45}Ca^{2+}$ appeared to be a true consequence of the activation of neutrophils with fMLP, as the unstimulated control did not show a marked increase in intracellular $^{45}Ca^{2+}$ levels. The mean uptakes of $^{45}Ca^{2+}$ by fMLP-activated control and moxifloxacin-treated neutrophils were 526 ± 35 and 570 ± 32 pmol$^{45}Ca^{2+}$ /10^7 cells respectively, measured 5 min after addition of fMLP when influx is completed (Anderson and Goolam Mahomed, 1997).

Measurement of the effects of moxifloxacin on pneumolysin-mediated influx of $^{45}Ca^{2+}$ into human neutrophils revealed that moxifloxacin (10 µg/ml) did not affect the pore-forming interactions of pneumolysin (8.37 ng/ml) with neutrophils. The mean uptakes of $^{45}Ca^{2+}$ were 151 ± 7 for the pneumolysin-free control system, and 2538 ± 60 and 2494 ± 113 pmol$^{45}Ca^{2+}$ /10^7 cells for the pneumolysin-treated systems in the absence and presence of moxifloxacin respectively. To confirm that moxifloxacin does not affect calcium fluxes, additional experiments were performed

to determine the effects of varying concentrations of moxifloxacin (2.5 – 20 µg/ml) on the spontaneous influx of $^{45}Ca^{2+}$ into unstimulated neutrophils. At all concentrations tested, moxifloxacin did not affect spontaneous Ca^{2+} uptake. The mean peak values were 667 ± 22 for resting cells and 684 ± 17, 667 ± 21, 648 ± 26 and 711 ± 53 pmol/10^7 cells for moxifloxacin (2.5, 5, 10 and 20 µg/ml)-treated neutrophils.

3.3.4.2 Effects on efflux of $^{45}Ca^{2+}$

In these experiments, neutrophils, which were pre-loaded with $^{45}Ca^{2+}$ (2 µCi/ml), washed and transferred to Ca^{2+}-replete HBSS, were activated with fMLP (1 µM) in the presence and absence of moxifloxacin (10 µg/ml), added 1 min prior to fMLP, followed by measurement of cell-associated $^{45}Ca^{2+}$. Moxifloxacin did not affect the magnitude of efflux of $^{45}Ca^{2+}$ from fMLP-activated neutrophils. The amounts of $^{45}Ca^{2+}$ discharged from unstimulated neutrophils were 567 ± 22 as compared to 1686 ± 34 and 1629 ± 29 pmoles/10^7 cells discharged from fMLP-activated control and moxifloxacin-treated neutrophils, respectively.

3.1 LUCIGENIN-ENHANCED CHEMILUMINESCENCE

Table 3.1a: Effects of moxifloxacin (1 - 20 µg/ml) on superoxide production by fMLP-activated neutrophils.

System	Superoxide production (r.l.u)
Resting cells	820 ± 58
fMLP control	3891 ± 563
fMLP + Moxifloxacin 1 µg/ml	3883 ± 550
fMLP + Moxifloxacin 2.5 µg/ml	3967 ± 577
fMLP + Moxifloxacin 5 µg/ml	3577 ± 547
fMLP + Moxifloxacin 10 µg/ml	3796 ± 472
fMLP + Moxifloxacin 20 µg/ml	4211 ± 456

The results of 10 experiments are presented as the mean absolute peak values ± SEMs measured 1 min after the addition of fMLP.

Table 3.1b: Effects of moxifloxacin (1 - 20 µg/ml) on superoxide production by PMA-activated neutrophils.

System	Superoxide production (mV/s)
Resting cells	485 ± 82
PMA control	3379 ± 195
PMA + Moxifloxacin 1 µg/ml	3504 ± 188
PMA + Moxifloxacin 2.5 µg/ml	3539 ± 228
PMA + Moxifloxacin 5 µg/ml	3856 ± 340
PMA + Moxifloxacin 10 µg/ml	3456 ± 221
PMA + Moxifloxacin 20 µg/ml	3367 ± 194

The results of 13 experiments are presented as the mean absolute peak values ± SEMs measured 10 min after the addition of PMA.

3.2 LUMINOL-ENHANCED CHEMILUMINESCENCE

Table 3.2a: Effects of moxifloxacin (1 - 20 µg/ml) on production of oxidants by the MPO/H_2O_2/halide system following activation of neutrophils with fMLP.

System	Superoxide production (r.l.u)
Resting cells	3142 ± 211
fMLP control	35898 ± 7256
fMLP + Moxifloxacin1 µg/ml	33838 ± 6574
fMLP + Moxifloxacin 2.5 µg/ml	33965 ± 6095
fMLP + Moxifloxacin 5 µg/ml	34069 ± 6291
fMLP + Moxifloxacin 10 µg/ml	34691 ± 6325
fMLP + Moxifloxacin 20 µg/ml	34711 ± 4859

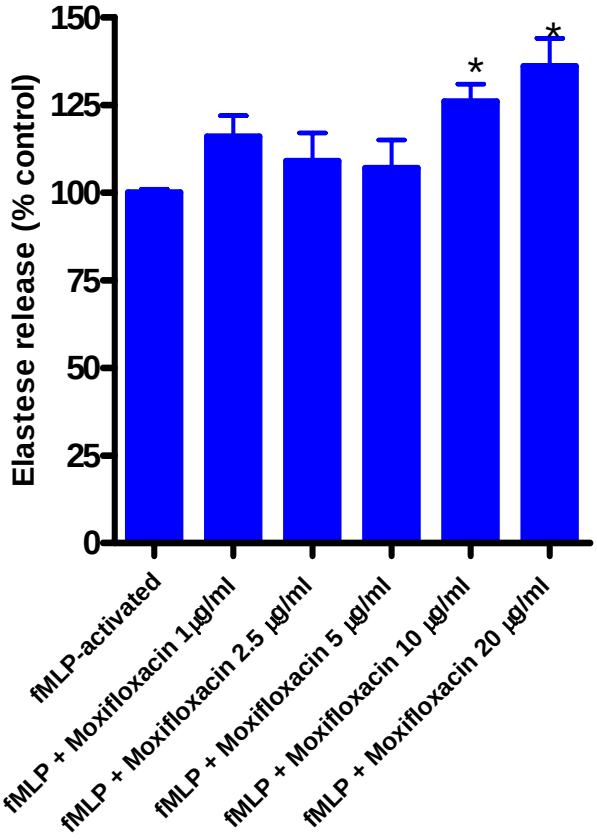
The results of 3 experiments are presented as the mean absolute peak values ± SEMs measured 1 min after the addition of fMLP.

Table 3.2b: Effects of moxifloxacin (1 - 20 µg/ml) on production of oxidants by the MPO/H_2O_2/halide system following the activation of neutrophils with PMA.

System	Superoxide production (mV/s)
Resting cells	1804 ± 82
PMA control	23938 ± 1946
PMA + Moxifloxacin 1 µg/ml	22108 ± 1770
PMA + Moxifloxacin 2.5 µg/ml	21217 ± 1656
PMA + Moxifloxacin 5 µg/ml	20868 ± 1680
PMA + Moxifloxacin 10 µg/ml	21040 ± 1816
PMA + Moxifloxacin 20 µg/ml	19273 ± 1521

The results of 3 experiments are presented as the mean absolute peak values ± SEMs measured 10 min after the addition of PMA.

Fig 3.1: Effects of Moxifloxacin on the release of elastase by fMLP/CB-activated neutrophils.



The results of 4 experiments are presented as the mean values ± SEMs for elastase measured in the supernatants of control and moxifloxacin-treated neutrophils 15 min after the addition of fMLP/CB.

3.4 DISCUSSION

Cellular signalling events leading to systemic inflammation are complex. It is generally accepted that recruitment and activation of leukocytes contribute to tissue damage in inflammation (Lucas *et al.,* 2003). Neutrophils migrate to the site of inflammation, where they are activated by different stimuli, resulting in the generation of large amounts of ROS and the release of granular enzymes such as elastase and myeloperoxidase (Choi *et al.,* 2003), which participate in tissue injury. There are a multitude of intracellular processes, which are linked to, or precede the production of inflammatory mediators (Hirayama *et al.,* 2000).

It has been highlighted recently that several fluoroquinolones, including moxifloxacin play an important role in host defence by priming (sensitizing) mature human phagocytes such as neutrophils and mononuclear leukocytes. For example, the ability of fMLP and PMA to stimulate superoxide production is significantly enhanced by pre-incubation of neutrophils with several fluoroquinolones (Niwa *et al.,* 2002; Dalhoff and Shalit, 2003). However, relatively little is known about the effect of moxifloxacin on host defences, particularly innate protective mechanisms involving neutrophils.

Lucigenin and luminol chemiluminescence measurements were used to evaluate the production of superoxide and HOCl respectively during neutrophil activation. Using both lucigenin- and luminol-chemiluminescence systems, moxifloxacin was found to have no significant priming or inhibitory effects on oxidant generation by human neutrophils stimulated with either PMA (which acts via a cascade involving protein kinase C) or fMLP (via a G-protein-coupled receptor) (Liao *et al*., 2005).

Some preliminary studies suggest that neutrophils kill ingested microorganisms by subjecting them to high concentrations of highly toxic ROS and bring about myeloperoxidase (MPO)-catalysed halogenation. Studies performed by Reeves and colleagues (2002) suggest that ROS generation and MPO activity are not themselves sufficient to kill microorganisms, instead ROS and cytoplasmic granules released into the phagocytic vacuole in which the microbes are encapsulated act together to destroy

internalized microbes.

When neutrophils are activated, receptors, adhesion molecules, and enzymes are translocated to the cell surface from intracellular granules, and some of these factors such as elastase (Mayer-Scholl *et al.,* 2004) and MPO (Topham *et al.,* 1998) are secreted. The process of degranulation influences neutrophil functional responses including adhesion to surfaces, aggregation, motility, and microbial killing. In subjects with granule deficiencies or impaired degranulation mechanisms, these events are altered and host defense is compromised (Tophan *et al*., 1998). In this part of my study, neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophil elastase is a neutrophil-derived toxic molecule that has been considered crucial in the pathophysiology of acute inflammatory responses (Kawabata *et al.,* 2002; Korkmaz *et al.,* 2005). Interestingly, moxifloxacin treatment at higher concentrations (10 and 20 μg/ml) showed a significant enhancement of elastase release by human neutrophils following activation with fMLP/CB for 15 min, suggesting that moxifloxacin at higher concentrations enhances neutrophil degranulation. Importantly, elastase, as well as other neutrophil granule serine proteases possesses antimicrobial activity (Reeves *et al.,* 2002), suggesting that moxofloxacin, albeit at relatively high concentrations, has potentiate the bactericidal actions of neutrophils.

Since degranulation by activated neutrophils is a Ca^{2+}-dependent process (Nüsse *et al.,* 1997; Ramafi *et al.,* 1999), the biochemical processes which mediate an increase in $[Ca^{2+}]_{i,}$ as well as those which restore Ca^{2+} homeostasis, were identified as possible targets of moxifloxacin. Increases in the concentrations of cytosolic Ca^{2+} are a powerful stimulus to cell activation (Li, 1998; Lucas, *et al.,* 2003). In inflammatory cells such as neutrophils or macrophages, in which Ca^{2+} influx is not mediated by voltage activated channels (Hallett, 2003), receptor mediated and store-operated Ca^{2+} influx pathways appear to be important mechanisms for calcium entry (Patterson and Rossum, 1999).

However, the results of the experiments using the radiometric procedures demonstrated that neither the efflux (an indirect measurement of the abruptly

occurring release of Ca^{2+} from intracellular stores) nor the store-operated influx of Ca^{2+} was affected by moxifloxacin. These results indicate that moxifloxacin alone does not induce movement of Ca^{2+} into or out of neutrophils, neither does it affect the magnitude of efflux or store-operated influx following activation of neutrophils with fMLP. Taken together, these observations clearly demonstrate that moxifloxacin does not affect the fMLP-receptor-G-protein interactions that lead to the activation of phospholipase C which in turn mediate generation of IP_3 in fMLP-activated neutrophils.

Notwithstanding complement-activating properties, pore-forming interactions with neutrophils and monocytes, resulting in influx of Ca^{2+}, have been implicated in the pro-inflammatory activities of the pneumococcal toxin, pneumolysin (Cockeran *et al.*, 2003). Rather than contributing to the eradication of the infection, however, the resultant, predominantly neutrophil-mediated inflammatory response appears to favour persistence and extrapulmonary dissemination of the pneumococcus (Cockeran *et al.*, 2003). In the current study, moxifloxacin did not affect pneumolysin-mediated influx of Ca^{2+}, indicating that moxifloxacin neither antagonizes nor potentiates the pore-forming activities of pneumolysin. Although antagonism of pneumolysin would be a beneficial property of antibiotics used in the treatment of pneumococcal diseases, moxifloxacin appears to be neutral in this respect.

Some preliminary studies reviewed by Dalhoff and Shalit (2003) suggest that fluoroquinolones interact directly with bacteria to inhibit adherence to and colonization of epithelial surfaces, reducing the release of pro-inflammatory bacterial products, while increasing uptake by phagocytosis and intracellular killing. In the case of the quinolones ofloxacin, ciprofloxacin, sparfloxacin and temafloxacin, Aoki *et al.*, (1994) found that these agents differ in their immunomodulating effects. For ofloxacin as well as ciprofloxacin, they found enhancement of the oxidative burst, but in contrast, inhibitory effects on the oxidative burst were observed for sparfloxacin and temafloxacin.

The results of the current study are in agreement with those of Fischer and Adam (2001) who demonstrated that moxifloxacin does not affect oxidative burst activity. I have found that moxifloxacin at therapeutically relevant concentrations and higher does not directly antagonize or enhance phagocytic functions, since it has little or no direct effects on the pro-inflammatory responses of human neutrophils. These observations are compatible with a mechanism whereby moxifloxacin potentiates host defenses indirectly by weakening bacteria, preventing their adherence to host tissues, while increasing susceptibility to phagocytosis and intracellular killing, as opposed to direct immunomodulatory interactions with neutrophils.