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In vitro anti-inflammatory effects and immunomodulation by gemifloxacin in stimulated human THP-1 monocytes

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Cultured human THP-1 monocytes were exposed to serial concentrations of gemifloxacin over 4 h after pre-stimulation with zymogen A for 1 h or *Staphylococcus aureus* for 2 h. The following parameters were assessed: pH, phagocytosis, c-AMP, NO, TNFα, IL-1, IL-6, IL-8 and H_2O_2 levels, enzyme activities of protein kinase C, NADPH oxidase, SOD, gluthathion reductase, NAG and cathepsin D as well as lipid peroxidation. The reversiblity of these changes was determined in the presence of known blockers of the phagocytic process. The effects of gemifloxacin on DNA synthesis and killing of *S. aureus* was assessed in bacteria alone and in those bacteria phagocytosed by THP-1 monocytes over 24 h. Gemifloxacin in stimulated THP-1 monocytes over the first 30 min caused an increase in c-AMP, NO, H_2O_2 and TNFα levels and protein kinase C, NADPH oxidase, glutathione reductase, NAG and cathepsin D activities. The pH became more acidic and phagocytosis was stimulated. These parameters were reversed at 1 h and continued to decline until 4 h. Lipid peroxidation was at the highest levels at 1 h and IL-8 levels at 2 h. DNA synthesis and bacterial growth were suppressed at 2 h in both *S. aureus* alone and bacteria phagocytosed by THP-1 monocytes. These effects were at a higher magnitude at 24 h. Gemifloxacin initiates a phagocyticidal effect of THP-1 monocytes at an early time of 30 min which plays a role in killing bacteria but a higher magnitude of killing of bacteria occurs later by a standard static mechanism. This early action of gemifloxacin should decrease the spread of infection and the inflammatory response since the tissue destruction process was attenuated at 4 h.

1. Introduction

It has been suggested that the macrolides and azalides in addition to their antibiotic activity also have anti-inflammatory and immunomodulation activity. Not only are these activities important in the actual killing of the invading pathogtosed bacteria, these properties of the drugs would be important in the spread of the infection and the subsequent damage to tissue. Recently it has been observed that a number of fluoroquinolones stabilize the lysosomal membranes blocking the release of hydrolytic enzymes (Carevic and Djokic 1988) and prevent the release of reactive oxygen species (Levert et al. 1988) and superoxide (Anderson et al. 1996). The macrolides modulate the release of TNFα, IL-1, IL-6 and IL-8 from stimulated neutrophils, macrophages and monocytes *in vitro* and *in vivo* varying over time as to when the cytokine level was elevated or suppressed (Khan et al. 1998, 1999; Bailly et al. 1990a, 1990b, 1991; Iino et al. 1992; Roche et al. 1987a, 1987b; Takeshita et al. 1989; Morikawa 1993a, 1993b, 1994, 1996; Ives et al. 2001).

The fluroroquinlones have demonstrated superior and wide antibiotic activity against the growth of pathogenic organisms that infect man. These antibiotics are unique in that they are taken up by phagocytosis into macrophages, monocytes or PMNs. The intracellular phagocytic vacuoles merge with lysosomes with the release of hydrolytic enzymes which kill the bacteria. Gladue et al. (1989a, 1989b) has suggested that plasma cells concentrate these antibiotic and act as vehicle to conduct the antibiotic to the site of inflammation in the body to suppress the infection. Human monocytes have been shown to concentrate sufficient quantities of azithrimycin (Hall et al. 2002), clarithromycin (Ives et al. 2001), moxifloxacin (Hall et al. 2003a), alatrofloxacin (Hall et al. 2003b) and grepafloxacin (Ives et al. 2003) to not only kill bacteria but to cause significant impact on cytokines and inflammatory mediator levels as well as tissue destructive enzymes.

Gemifloxacin (SB-265805, LB20304a) is a novel pyrrolidine containing fluoronaphthyridone carboxylic acid with broad-spectrum antibiotic activity against Gram-positive and Gram-negative pathogens (Cormican and Jones, 1997; Goldstein et al. 1997; Mortensen and Rodges, 2000; McCloskey et al. 2000; Martinez-Martinez et al. 2001; Jorgensen et al. 2000, Lopez et al. 2001; Kerawala et al.

2001; Nagai et al. 2001; Hoban et al. 2001; McGowen et al. 2001; Thadepalli and Reddy, 1997; Girard and Girard, 1995) with select activity against anaerobic organisms (Kleinkauf et al. 1996, 2001). Especially potent activity of gemifloxacin was observed against Gram-positive organisms (Nagai et al. 2001) demonstrating improved activity over ciprofloxacin and trovafloxacin and resistant strains to penicillin, glycopeptides and other flurorqinolidones (Jorgensen et al. 2000; Lopez et al. 2001; Kerawala et al. 2001). This antibiotic had been shown to be taken up by macrophages (Edelstein et al. 2001) and to inhibit two enzymatic targets: the bacterial gyrase and DNA topoisomerase IV (Heaton et al. 2000, Hooper, 2001). Dual target of an antibiotic is an advantage considering the propensity for bacteria to develop resistance to antibiotics. Mutation of one target enzyme most probably does not impact the remaining target leaving it intact for inhibition by the antibiotic so that antibacterial action is not totally lost.

The present study involves the examination of the effect of gemifloxacin in stimulated human phagocytic THP-1 monocytes on cytokine and chemical mediator release and the inflammatory process.

2. Investigations and results

2.1. Oxygen burst

The initiation of the oxygen burst during phagocytosis occurs through membrane stimulation and the activation of NADPH oxidase and protein kinase activities with release of c-AMP and chemical mediators. Incubation of zymogen A stimulated THP-1 monocytes with gremifloxacin led to an increase in c-AMP levels after 15 min and c-AMP remained elevated for 45 min (Table 1). This effect on c-AMP levels was concentration dependent. Protein kinase C activity was elevated at 4 μg/mL at 15 and 30 min returning to normal levels at 1 h. NADPH oxidase activity was elevated over the first hour reaching a peak at 30 min with the effect appearing concentration dependent. NO release from stimulated THP-1 monocytes was significantly elevated for 1 h at 4 and 0.4 μg/mL but reached values below normal at 2 h. Hydrogen peroxide release was also elevated reaching a peak at 30 min. At the highest concentrations, at 4 and 0.4 μg/mL, the release of hydrogen peroxide remained significantly higher for 2 h. SOD activity was not significantly elevated over the 4 h period but glutathione reductase activity was sig-

Table 1: Effects of gemifloxacin on human THP-1 monocyte phagocytosis processes at pH 7.4

Assay N = 6	H	Control %	4 μg/mL	0.4 μg/mL	0.04 μg/mL	0.004 μg/mL
c-AMP	0.25	100 ± 5	312 ± 7*	280 ± 6*	274 ± 6*	272 ± 7*
c-AMP	0.5	100 ± 5	161 ± 5*	156 ± 4*	154 ± 4*	137 ± 5*
c-AMP	0.75	100 ± 4	150 ± 3*	149 ± 4*	125 ± 3*	109 ± 4
c-AMP	1	100 ± 3	114 ± 4	105 ± 3	102 ± 4	90 ± 6
Protein Kinase C	0.25	100 ± 3	131 ± 4*	117 ± 4*	105 ± 3	105 ± 3
Protein Kinase C	0.30	100 ± 4	132 ± 3*	112 ± 3	109 ± 4	87 ± 3
Protein Kinase C	1	100 ± 4	96 ± 3	76 ± 4*	75 ± 3*	59 ± 4*
NADPH Oxidase	0.25	100 ± 3	116 ± 3*	114 ± 4	113 ± 5	111 ± 4
NADPH Oxidase	0.5	100 ± 4	150 ± 5*	143 ± 5*	127 ± 3*	106 ± 5
NADPH Oxidase	1	100 ± 5	112 ± 4	111 ± 3	105 ± 5	102 ± 4
NADPH Oxidase	2	100 ± 5	105 ± 3	103 ± 6	86 ± 5	85 ± 4
NADPH Oxidase	4	100 ± 4	85 ± 4	68 ± 4*	67 ± 5*	58 ± 5*
NO Release	0.25	100 ± 4	190 ± 5*	188 ± 7*	122 ± 4*	93 ± 5
NO Release	0.5	100 ± 4	150 ± 6*	140 ± 5*	103 ± 5	53 ± 4*
NO Release	1	100 ± 4	135 ± 5*	123 ± 4*	115 ± 3	33 ± 5*
NO Release	2	100 ± 3	66 ± 4*	56 ± 5*	54 ± 3*	30 ± 2*
NO Release	4	100 ± 5	61 ± 5*	61 ± 3*	60 ± 3*	16 ± 2*
H_2O_2 Release	0.25	100 ± 3	140 ± 4*	126 ± 3*	125 ± 3*	109 ± 4
H_2O_2 Release	0.50	100 ± 4	231 ± 5*	148 ± 4*	147 ± 5*	120 ± 4*
H_2O_2 Release	1	100 ± 5	166 ± 4*	107 ± 6	106 ± 5	96 ± 4
H_2O_2 Release	2	100 ± 3	131 ± 4*	100 ± 4	92 ± 5	89 ± 3
H_2O_2 Release	4	100 ± 4	112 ± 4	99 ± 5	91 ± 6	62 ± 4
SOD	0.25	100 ± 3	107 ± 5	105 ± 6	92 ± 5	90 ± 5
SOD	0.5	100 ± 4	109 ± 6	97 ± 4	96 ± 2	93 ± 5
SOD	1	100 ± 4	103 ± 4	102 ± 5	98 ± 5	92 ± 6
SOD	2	100 ± 2	105 ± 6	102 ± 4	98 ± 3	89 ± 4
SOD	4	100 ± 4	88 ± 4	82 ± 5	81 ± 4	80 ± 3
Glutathione reductase	0.25	100 ± 4	339 ± 9*	193 ± 6*	159 ± 5*	111 ± 3*
Glutathione reductase	0.5	100 ± 4	114 ± 3	109 ± 5	90 ± 5	88 ± 7
Glutathione reductase	1	100 ± 5	94 ± 5	92 ± 4	91 ± 6	81 ± 2
Glutathione reductase	2	100 ± 3	40 ± 2*	37 ± 4*	36 ± 3*	29 ± 3*
Cell pH	0.25	100 ± 2	114 ± 2*	113 ± 3*	113 ± 3*	101 ± 2
Cell pH	0.5	100 ± 3	112 ± 2*	111 ± 3*	105 ± 3	104 ± 3
Cell pH	1	100 + 3	106 ± 5	101 ± 4	98 ± 4	96 ± 3
Cell pH	2	100 ± 4	104 ± 6	101 ± 5	96 ± 3	99 ± 4
Cell pH	4	100 ± 3	102 ± 4	99 ± 6	92 ± 4	89 ± 3
Phagocytosis	0.25	100 ± 5	163 ± 5*	160 ± 4*	159 ± 4*	158 ± 4*
Phagocytosis	0.5	100 ± 2	159 ± 4*	154 ± 5*	153 ± 5*	151 ± 4*
Phagocytosis	1	100 ± 4	110 ± 4	105 ± 5	104 ± 6	103 ± 5
Phagocytosis	2	100 ± 3	102 ± 4	99 ± 5	98 ± 5	97 ± 3
Phagocytosis	4	100 ± 4	101 ± 5	95 ± 4	93 ± 4	90 ± 2

* p 0.001

Table 2: Effects of gemifloxacin on cytokine levels after previous treatment of human THP-1 monocytes with zymogen A for 1 h

Assay N = 6	Time (h)	Control %	4 μg/mL	0.4 μg/mL	0.04 μg/mL	0.004 μg/mL
TNFα	0.5	100 ± 4	312 ± 6*	280 ± 8*	274 ± 6*	272 ± 8*
TNFα	1	100 ± 3	161 ± 6*	156 ± 4*	154 ± 5*	138 ± 4*
TNFα	2	100 ± 3	150 ± 4*	149 ± 5*	125 ± 3*	109 ± 5
TNFα	4	100 ± 5	114 ± 4*	105 ± 5*	102 ± 5	90 ± 4
IL-1	0.5	100 ± 2	94 ± 5	93 ± 3	83 ± 6	80 ± 4
IL-1	1	100 ± 4	103 ± 6	104 ± 5	92 ± 3	88 ± 7
IL-1	2	100 ± 3	86 ± 5	76 ± 6*	73 ± 3*	71 ± 5*
IL-6	2	100 ± 3	96 ± 4	86 ± 2	83 ± 5	71 ± 3*
IL-6	4	100 ± 4	88 ± 3	88 ± 4	81 ± 3*	79 ± 2*
IL-8	1	100 ± 2	145 ± 5*	146 ± 4*	140 ± 5*	126 ± 4*
IL-8	2	100 ± 4	384 ± 3*	373 ± 6*	335 ± 5*	281 + 4*
IL-8	4	100 ± 5	346 ± 6*	186 ± 4*	186 ± 5*	180 ± 3*

Table 3: Effects of gemifloxacin on lysosomal enzymes and lipid peroxidation after pretreatment of THP-1 monocytes with zymogen A for 1 h

Assay N = 6	Hour	Control %	4 μg/mL	0.4 μg/mL	0.04 μg/mL	0.004 μg/mL
NAG	0.25	100 ± 4	221 ± 5*	135 ± 5*	116 ± 4	94 ± 5
NAG	0.5	100 ± 3	257 ± 4*	197 ± 5*	163 ± 4*	128 ± 6*
NAG	1	100 + 4	147 ± 5*	99 ± 5	98 ± 4	93 ± 6
NAG	2	100 ± 2	95 ± 6	94 ± 3	91 ± 2	87 ± 5
NAG	4	100 ± 3	95 ± 4	73 ± 4*	73 ± 3*	68 ± 4*
Cathepsin D	0.25	100 ± 4	143 ± 4*	127 ± 3*	114 ± 4	106 ± 3
Cathepsin D	0.5	100 ± 3	174 ± 5*	165 ± 4*	132 ± 4*	130 ± 5*
Cathepsin D	1	100 ± 6	135 ± 4*	132 ± 3*	130 ± 5*	96 ± 4
Cathepsin D	2	100 ± 5	133 ± 4*	116 ± 5	110 ± 3	75 ± 4*
Cathepsin D	4	100 ± 4	106 ± 5	103 ± 6	99 ± 4	95 ± 5
Cathepsin D	6	100 ± 4	103 ± 5	88 ± 5	80 ± 4*	74 ± 3*
Lipid peroxidation	0.25	100 ± 3	119 ± 3*	113 ± 5	110 ± 4	106 ± 5
Lipid peroxidation	0.5	100 ± 5	126 ± 5*	118 ± 4*	116 ± 5	110 ± 4
Lipid peroxidation	1	100 ± 2	165 ± 4*	153 ± 5*	145 ± 6*	136 ± 3*
Lipid peroxidation	2	100 ± 3	139 ± 3*	112 ± 3	109 ± 7	108 ± 3
Lipid peroxidation	4	100 ± 4	128 ± 5*	91 ± 6	86 ± 5	75 ± 6*

N = 6 * p = 0.001

nificantly increased after 15 min of drug exposure in a concentration manner. THP-1 cell pH after the addition of gemifloxacin was more basic than that of untreated cells, but returned to normal, pH 7.4, over the 4 h period. Phagocytosis was elevated at 15 and 30 min but returned to normal levels after 1 h.

2.2. Cytokines

Examination of the cytokine levels showed that TNFα levels were increased in a concentration dependent manner at 0.5, 1 and 2 h (Table 2). IL-8 levels were elevated at 1, 2 and 4 h with the highest levels being at 2 h and the effect was concentration dependent. IL-1 and IL-6 levels were not elevated, but the IL-1 levels were significantly reduced at 2 h and IL-6 levels were reduced at 2 and 4 h when lower concentrations of the drug were tested.

2.3. Hydrolytic enzymes

Lysosomal NAG activity in stimulated THP-1 monocytes was elevated at 15 and 30 min in a concentration manner but after 4 h the NAG activity was reduced below normal levels (Table 3). Cathepsin D activity was elevated over the first 2 h but again returned to normal levels by 4 h. Lipid peroxidation at 4 h at 4 μg/mL of drug was elevated from 15 min to 1 h and then returned to normal levels at the lower concentrations of gemifloxacin. Similar observations were also made with THP-1 monocytes stimulated with *S. aureus* for 2 h where at the early hours pH, NAG activity and hydrogen peroxide release are elevated and then over time these parameters return to normal or lower levels than observed in untreated cells (Fig. 1).

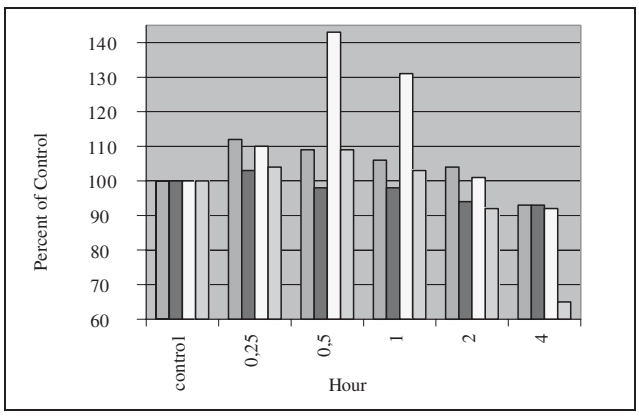

Fig. 1: Effects of gemifloxacin at 4 μg/mL over 4 h on pH, phagocytosis, hydrogen peroxide and NAG activity after 2 h pretreatment of THP-1 monocytes with *S. aureus*; N = 4, standard deviation were all within 2.2%; ■ pH; ■ Phagocytosis; □ H_2O_2; ■ NAG

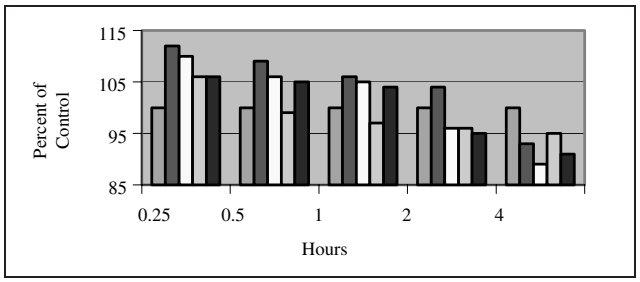

Fig. 2: Effects of gemifloxacin on the pH of zymogen stimulated THP-1 monocytes after 1 h in the presence of inhibitors of the phagocytic process; N = 6 Standard Deviations were all within 2.5%; ■ Control; ■ Gemif; □ NaF; ■ NH_4Cl; ■ CCCP

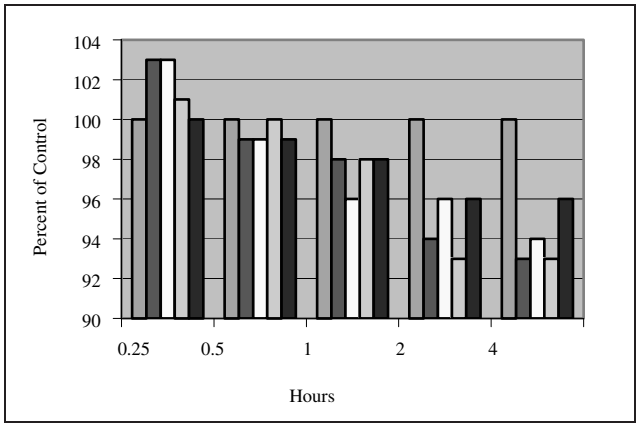

Fig. 3: Effects of gemifloxacin on phagocytosis of zymogen A stimulated THP-1 monocytes after 1 h in the presence of inhibitors of the phagocytic process; N = 6 Standard Deviations were all within 3.7%; ■ Control; ■ Gemif; □ NaF; ■ NH_4Cl; ■ CCCP

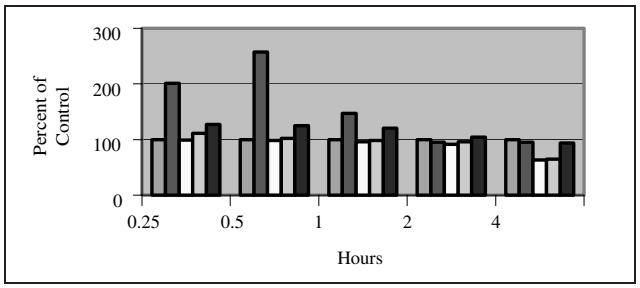

Fig. 4: Effects of gemifloxacin on NAG activity of zymogen A stimulated THP-1 monocytes after 1 h in the presence of inhibitors of the phagocytic process; N = 6 Standard Deviations were all within 4.3%; ■ Control; ■ Gemif; □ NaF; ■ NH_4Cl; ■ CCCP

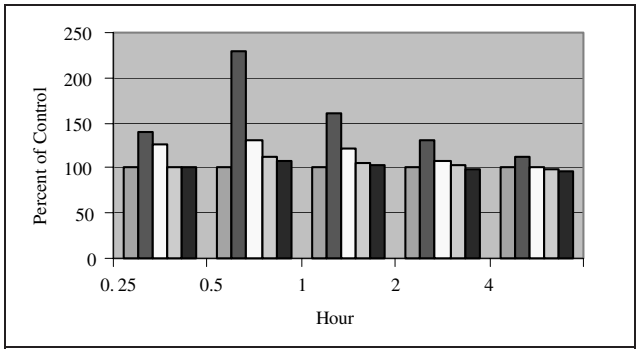

Fig. 5: Effects of gemifloxacin on hydrogen peroxide release of zymogen A stimulated THP-1 monocytes after 1 h in the presence of inhibitors of the phagocytic process; N = 6 Standard Deviations were all within 3.9%; ■ Control; ■ Gemif; □ NaF; ■ NH_4Cl; ■ CCCP

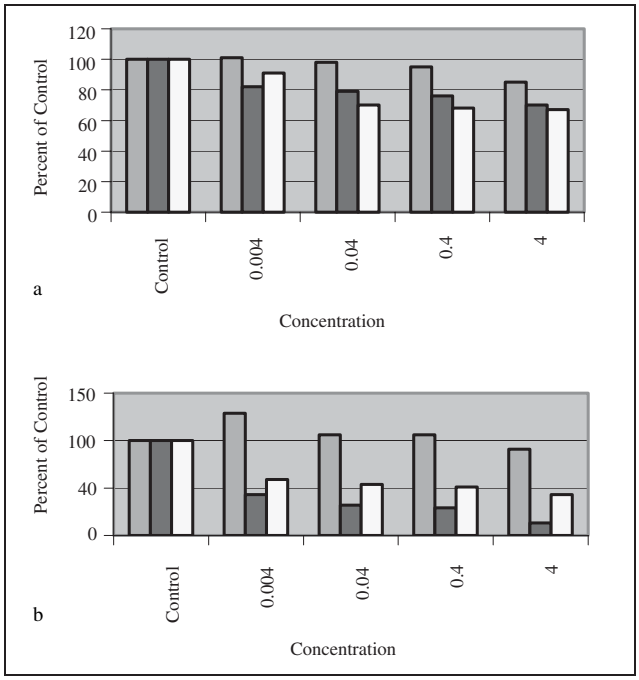

Fig. 6: Effects of gemifloxacin on DNA synthesis of THP-1 monocytes, *S. aurens* and phagocytosed bacteria at 2 and 24 h; N = 6 Standard Deviations (a) were all (b) within 5.1%; ■ THP-1; ■ S. aureus; □ THP-1 + S. aureus

2.4. Co-incubation with inhibitors

Co-incubation of zymogen A stimulated THP-1 monocytes with gemifloxacin and inhibitors of energy for phagocytosis, blocker of phagocytosis and lysosomal vacuoles or blocker of the membrane proton pump showed that these agents suppressed the increase in pH (Fig. 2), the increases in phagocytosis (Fig. 3) and NAG activity (Fig. 4) and the release of hydrogen peroxide (Fig. 5) induced by gemifloxacin at 4 μg/mL over 4 h.

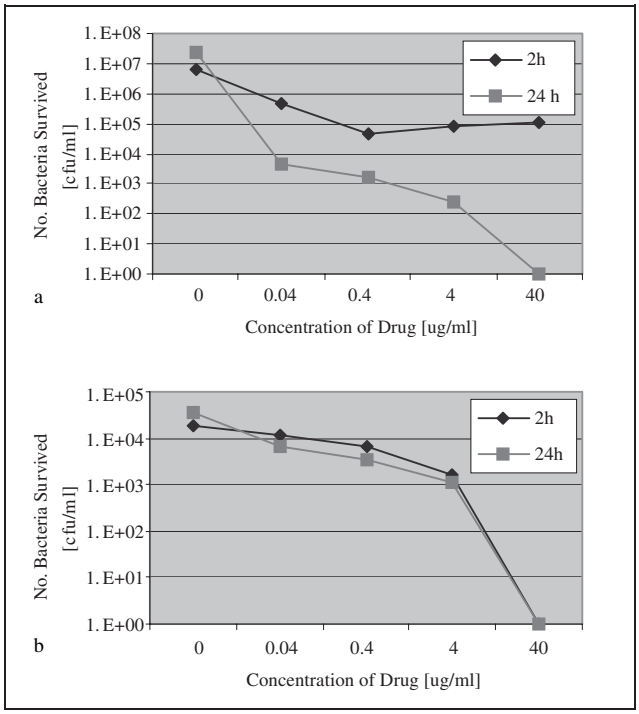

Fig. 7: Effect of gemifloxacin on *S.aureus* at 2 and 24 h without (a) and with (b) the presence of human THP-1 monocytes

2.5. Inhibition of DNA synthesis and bacterial cell death

DNA synthesis was not significantly inhibited in THP-monocytes incubated with gemifloxacin at 4 μg/mL for 2 or 24 h (Fig. 6). DNA synthesis in *S. aureus* incubated with gemifloxacin was inhibited slightly at 2 h in a concentration dependent manner but was more drastically reduced at 24 h. Examination of DNA synthesis in the *Staphylococci aurei* phagocytosed by THP-1 monocytes was reduced marginally at 2 h but more significantly reduced at 24 h in a concentration dependent manner.

Gemifloxacin decreased the number of S. aureus bacteria in a concentration dependent manner from 0 to 0.4 μg/mL at 2 h. At higher concentration at 24 h, a further reduction in cell number was observed reaching a total killing effect at 40 μg/mL of the drug. When the bacteria were phagocytosed by THP-1 monocytes the number of staphylococci decreased in a concentration dependent manner reaching total killing effect at 40 μg/mL at 2 and 24 h (Fig. 7).

3. Discussion

Gemifloxacin, a unique fluoroquinolone antibiotic, behaved in a similar manner as azithromycin, clarithromycin and moxifloxacin in stimulated THP-1 monocytes. The effects of antibiotic treatment occurred into two stages. First, in the presence of the antibiotic initially there was the release of c-AMP, NO, hydrogen peroxide and TNFα with the activation of protein kinase C, NADPH oxidase and lysosomal hydrolytic enzymes NAG and cathepsin D. All of these cellular events are associated with the uptake of foreign organisms by the phagocytosis process leading to a cidal killing of the organism. If this process is not controlled then inflammation, infections and immune responses would spread throughout the body. Second, elevation of GSH levels due to elevated glutathione reductase activity would neutralize the free radicals and there was a decrease in the lysosomal hydrolytic enzymes in the presence of the antibiotic. Lipid peroxidation was reduced at this later time and tissue destruction and spread of the infection should be reduced. IL-6 and IL-1, pro-inflammatory cytokines, which sustain the inflammation and immune response, are not elevated at this time in the presence of gemifloxacin and actually the release of these cytokines is reduced over time. IL-8 has been reported to be elevated at 4 mg/mL or lower in human alveolar macrophages after treatment with azithromycin or clarithromycin (Kurdowska et al. 2001). These authors suggested that high levels of IL-8, as a chemokine, would attract neutrophiles and other inflammatory cells to help fight the infection. Gemifloxacin significantly elevated IL-8 levels at a time between 2–4 h when the inflammation process was being suppressed in THP-1 monocytes.

DNA synthesis as a measure of growth in THP-1 monocytes was not affected significantly at 2 or 24 h in the presence of gemifloxacin. DNA synthesis in extracellular bacteria and those ingested by THP-1 monocytes was marginally inhibited at 2 h but DNA synthesis was more significantly inhibited at 24 h suggesting that bacteria sensitive to gemifloxacin were killed at these later times by a standard bacterial static mechanism.

4. Experimental

4.1. Source of materials

Gemifloxacin (SB 265805-S, LB-20304-A) was supplied by GlaxoSmithKline, Research Triangle Park, NC 27707). All other supplies unless otherwise noted were purchased from Sigma Chemical Co. (St. Louis, MO).

4.2. Cell culture techniques

THP-1 acute monocytes (ATCC TIB-202; American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI-1640 growth medium (GIBCO, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, VA, USA), and 3×10^{-5} M β-mercaptoethanol and penicillin (100 units/mL)/streptomycin (100 μg/mL) at 37 °C in a 5% CO_2 incubator (Ives et al. 2001). Cells were fed fresh growth medium 18 h without P/S antibiotics before each study.

4.3. Effects of gemifloxacin in zymogen A or bacteria stimulated THP-1 monocytes on metabolic events

THP-1 monocytes (10^6 cells) were pre-treated with zymogen A (0.5 mg/mL) for 1 h, or *S. aureus* for 2 h (bacteria : monocyte ratio of ~ 10 : 1) and then incubated with gemifloxacin at concentrations of 0.004, 0.04, 0.4 or 4 μg/mL in 96-well plates over time from 0–4 h after which a number of biochemical assays were performed (Ives et al. 2001). For determining changes in pH and phagocytosis these cell preparations were incubated with acridine orange (14.4 mg/100 mL) at pH 7.2 for 20 min and quenched with crystal violet (50 mg/100 mL) for 1 min. Using a Cytofluor 2350 Fluoresence Measurement System (Millipore Corp., Bedford, Mass.) with excitation at 450 nm, the cellular pH change from pH 7.4 (control value) was determined at 520 nm and phagocytosis was determined at 620 nm (Delic et al. 1991; Golder et al. 1983). NADPH oxidase activity was determined as the rate of cytochrome C reduction at 550 nm (Styrt and Klempner 1986). Protein kinase C activity was determined via ELISA immunoassay techniques (kit # 539484; Calbiochem, San Diego, Cal.). Nitric oxide (NO) release was determined spectrophotometrically (kit # 482650; Calbiochem) and read at 560 nm. Hydrogen peroxide release was measured with a Bioxytech H_2O_2 -560 kit (Oxis International, Inc.) and read at 560 nm. Glutathione reductase activity was determined spectrophotometrically by measuring the oxidation of NADPH at 340 nm (kit # 359962; Calbiochem). Superoxide dismutase (SOD) activity was assayed using a kit (# 574600; Calbiochem) and read at 490 nm. Cathepsin D activity was determined with ELISA kit (# QIA-29; Calbiochem). *N*-Acetyl glucosaminidase (NAG) activity was determined with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Ford-Hutchinson et al. 1984) as the substrate. The enzyme reaction was terminated with glycine buffer, pH 10.6 and read at 409 nm using a visible 96 well plate reader (Molecular Devices Corp., Sunnyvale, CA) with *p*-nitrophenol as the standard. Lipid peroxidation after antibiotic exposure was determined by a colorimetric method (Bioxytech LPO-586; Oxis International, Portland, OR.).

4.4. Effects of gemifloxacin on TNFα, IL-1, IL-6 and IL-8 release

After zymogen A stimulated THP-1 monocytes (10^6 cells/mL) were exposed to gemifloxacin at 0.004, 0.04, 0.4 or 4 μg/mL over 4 h, cell free extract were obtained and used to evaluate cAMP, TNFα, IL-1, IL-6 and IL-8 release via ELISA immunoassays (Quantikine kits # DE0450, DTA50, DLA50, D6050 and D1500, respectively from R & D Systems, Minneapolis, MN).

4.5. Co-Incubation studies with inhibitors of the phagocytic process

THP-1 monocytes (10^6 cells) were incubated with gemifloxacin at 4 μg/mL as well as one of the following agents: NaF at 10 μM which blocks glycolysis and the pentose phosphate shunt reducing energy for the phagocytic process, NH_4Cl at 10 mM which blocks the fusion of the phagasome and lysosome vacuoles and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at 50 μM a partial inhibitor of the pH gradient-activated chloride ion uptake of phagosomes (Ives et al. 2001). THP-1 monocytes pH, phagocytosis, NAG activity and hydrogen peroxide release were evaluated to determine if the effects of gemifloxacin on these parameters were reversed by these inhibitors over 4 h.

4.6. Inhibition of DNA synthesis after exposure to S. aureus for 2 or 24 h

Human THP-1 monocytes (10^6 cells) were pre-incubated with *S. aureus* (bacteria : monocyte ratio of ~ 10 : 1) for 2 h and non-phagocytosed bacteria were removed by lysostaphin treatment as described below. Gemifloxacin and 100 μL of [methyl–^{3}H]-thymidine (65.3 mCi/mmol) (Moravek Biochemicals, Brea CA., U.S.A.) were added and incubated for 2 or 24 h. The reaction was stopped with 10% percholic acid and the acid treated soluble precipitate was collected on GF/A filters (Fischer Scientific, Atlanta, Ga.) by vacuum suction, counted and corrected for quenching.

4.7. Intracellular and extracellular activity of gemifloxacin against S. aureus

Human THP-1 monocytes were stimulated with *S. aureus*, as described above. Then, non-ingested bacteria were removed by incubating the suspension with lysostaphin (10 μg/ml) for 15 min at 37 °C) Fietta et al. 1997). The suspension was then centrifuged and the monocytes with in-

gested bacteria were incubated in the absence (control) or presence of different concentrations of gemifloxacin. At this time, the number of monocyte-associated microorganisms was approximately 5×10^4 cfu/mL. After 2 and 24 h of incubation, samples were removed and the monocytes disrupted by brief sonication. Serial dilutions were performed and plated onto tryplicase soy agar to determine the number of viable intracellular bacteria. To assess the activity of gemifloxacin against *Staphylococci* in THP-1 cell-free medium, pre-opsonized *S. aureus* bacteria ($\sim 10^6$ cfu/mL) were exposed to the drug at 37 °C in the absence of monocytes. After 2 or 24 h, samples were removed and serial dilutions plated onto agar. The assay was repeated with differences between assays being < 1 log 10 cfu/mL. Fig. 7 is a representative example of the results.

4.8. Statistical analysis

Data are presented in the tables and figures as the percent of control with standard deviations. The probable significance difference between the control and treated raw data was determined by the Student's "t" test.

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