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## New Improved Quinolone Derivatives Against Infection

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### 1. Introduction

There had been a tremendous advancement in the quality and quantity of worldwide research production in the field of microbiology. Among various biomedical fields, microbiology has been the subject of extensive study due to the problems imposed on human health by countless infectious diseases known so far. Identification of infectious agents and to adapt measures for its eradication is considered a major tool for alleviating human from the burden of infections. Progressively it is important to modulate the structure of earlier marketed antibacterial agents to produce newer agents with superior antimicrobial profile.

Fluoroquinolones, useful in the treatment of many bacterial infections, attacks DNA gyrase and topoisomerase IV on bacterial chromosomal DNA (Ball et al., 1998, Blandeau, 1999). However, widespread use of fluoroquinolones has caused the resistant rates of various Gram-negative bacilli (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella*), to approach the critical points.

To solve the problem of increasing antimicrobial resistance, it is crucial to design and produce new quinolones that could provide effective therapy for infections caused by organisms resistant to older agents. Most of the quinolones currently on the market or those which are under development, displays only moderate activity against many Gram-positive cocci, including Staphylococci and Streptococci. This insufficient activity has not only limited their use in infections caused by these organisms, such as respiratory tract infections, but has also been believed to be one of the reasons for the rapidly developing quinolone resistance. Therefore, recent efforts have been directed toward the synthesis of new quinolone antibacterials that can provide improved Gram-positive antibacterial activity, while retaining good Gram-negative activity

The extensive research efforts have enabled a better definition of the structural moieties or elements around the basic pharmacophore of quinolones that offer the best combination of clinical efficacy and reduced resistance selection in Gram-positive and Gram negative bacteria.

The general structure of quinolone antibacterial agents consist of a 1-substituted-1,4 dihydro-4-oxopyridine-3-carboxylic moiety A combined with an aromatic or hetero-aromatic ring B.

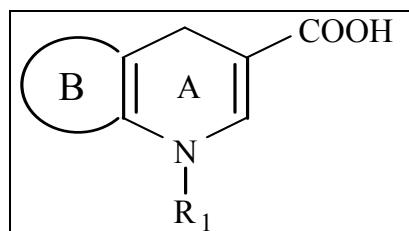


Fig. 1.

Most of the current agents have a carboxyl group at position 3, a keto group at position 4, a fluorine atom at position 6, and a nitrogen heterocycle moiety at the C-7 position (Sarro & Sarro, 2001). In addition at the C-7 position, substitution of bulky functional groups is permitted (Emami et al., 2006). The substituent at N-1 and C-8 positions of the quinolones should be relatively small and lipophilic to enhance self-association and also control potency and pharmacokinetics. Groups at C-5 and C-6 position controls potency for Gram-positive activity. SAR studies on quinolones explain that C-3 and C-7 positions are vital for antibacterial activities.

Most of the quinolone antibacterial research has been focused on the functionality at C-7 position as it is the most adaptable site for chemical change (Anderson & Osheroff, 2001, Foroumadi et al., 2005) **416**. The 3-carboxylic group is considered important for DNA gyrase binding (Chu et al., 1985, Domagala et al., 1988, Sarro & Sarro, 2001). Modifications on this position of quinolone are normally not accepted (Mitscher, 2005). However, there are some exceptions to this rule; of which, one was ester pro-drug analogues that were converted *in-vivo* back to the acid (Kondo et al., 1988). In one case, replacement of 3-carboxylic acid group of ciprofloxacin with bioisostere- fused isothiazolo ring was found more potent (4 to 10 times) than ciprofloxacin and possessed enhanced activity against DNA gyrase (Chu & Fernandes, 1989). A series of the C3/C3 bis-fluoroquinolones tethered with an 1, 3, 4-oxadiazole ring were reported showing antitumor activity (Hu et al., 2012) while 1,8 Imidazo fused quinolones exhibited moderate antibacterial activity (Venkat Reddy et al., 2009). Recently two naphthyl ester quinolone derivatives have been reported to demonstrate photo-oxidant properties (Vargas et al., 2008).

Advances in quinolone field are likely to provide better compounds capable of dealing with the resistant strains. These research efforts have been rewarded by very significant improvements in antibacterial potency as well as *in vivo* efficacy. It is evident from literature that most of the research on quinolone is directed towards group modification of the C-7 basic group of the quinolone and the effect of substitution at C-3 position of quinolones has not been studied to a large extent to produce new agents with better antimicrobial profile. Accordingly to explore the potential of 3-carboxylic quinolone derivatives as anti Gram positive and Gram negative agents, we have recently reported some novel enoxacin (Saeed Arayne et al., 2009) , levofloxacin (Sultana et al., 2011) and ofloxacin (Arayne et al., 2012) carboxamide analogues by introducing new functionality at C-3 position. In continuation of our research program to establish the structure-activity relationships of 3-carboxylic quinolone derivatives, we here in report some novel enoxacin analogues which have been prepared by introducing new functionality at C-3. These compounds were prepared by two series of reactions. In reaction **1**, the carboxylic group at C-3 position of enoxacin was esterified in methanol followed by reaction **2**, whereby the ester group was subjected to nucleophilic attack at the carbonyl carbon by various aliphatic (urea, thiourea, acetamide,

thioacetamide) and aromatic amines (phthalimide, benzamide) yielding amides and regenerating alcohols.

## 2. Experimental

### 2.1 Physical measurements

Melting points were obtained manually by capillary method. Infrared spectra were recorded on FT-IR spectrophotometer (shimadzu prestige-21 200 VCE coupled to a P IV- PC and loaded with IR solution version 1.2 software). The potassium bromide disks were placed in the holder directly in the IR laser beam. Spectra were recorded at a resolution of 2 cm<sup>-1</sup> and 50 scans were accumulated. NMR spectra were recorded on Bruker FT - NMR 500 MHz with the compounds dissolved in DMSO. Chemical shifts are reported in parts per million ( $\delta$ ) relative to tetra methyl silane as an internal standard. Significant <sup>1</sup>H NMR data are tabulated in the following order: number of proton(s) and multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet). The mass spectra were recorded on Finnign-MAT212 under electron impact (EI) ionization condition. Thin layer chromatography (TLC) was performed on HSF-254 TLC plate and compounds visualized under UV lamp.

### 2.2 Antibacterial studies

Disk Diffusion technique developed by Bauer et al (Baur et al., 1966) was adopted to determine the antibacterial activity of enoxacin and its derivatives against 10 different clinical isolates of Gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pneumoniae*) and Gram negative organisms (*Klebsiella Pneumoniae*, *Proteus mirabilis*, *Shigella flexneri*, *Escherichia coli*, *Pseudomonas areuginosa*, *Citrobacter species*, and *Salmonella typhi*).

50  $\mu\text{g mL}^{-1}$  stock solutions of the drug and their derivatives were prepared in hot methanol. The stock solution was diluted to 3 different concentrations i.e. 5, 10 and 20  $\mu\text{g mL}^{-1}$ . Commercially available filter paper discs were impregnated with the prepared solutions of the drug and the derivatives, dried and applied on the surface of the agar plate over which a culture of micro organism was already streaked. After 24 hours of incubation the clear zone of inhibition around the disc was determined, this is proportional to the bacterial susceptibility for the antimicrobial agent present in the disk. Three replicas were made for each treatment to minimize error.

### 2.3 Antifungal studies

Enoxacin and its derivatives were tested for antifungal activity against the fungi; *Aspergillus purasiticus*, *Saccharomyces cervics*, *Candida albicans* and *Fusraium solani*.

100  $\mu\text{g mL}^{-1}$  stock solutions of the compounds were prepared in hot methanol. The stock solutions were diluted to 3 different concentrations i.e. 30, 40 and 50  $\mu\text{g mL}^{-1}$ . Commercially available filter paper discs were impregnated with the prepared solutions of the drugs and the compounds under study, dried and applied on the surface of the agar plate over which a culture of micro organism was already streaked. After 24 hours of incubation the clear zone of inhibition around the disc was determined, this is proportional to the fungal susceptibility for the antimicrobial agent present in the disk. Three replicas were made for each treatment to minimize error.

## 2.4 Phagocyte chemiluminescence assay

The assay was performed as described by Helfand et al. protocol (Helfand et al., 1982). Briefly enoxacin and its derivatives were diluted in three concentrations, (0.5, 5 and 25  $\mu\text{g mL}^{-1}$ ) in Hanks Balanced Salt Solution containing calcium and magnesium (HBSS<sup>++</sup>). 25  $\mu\text{L}$  diluted blood (1:50 dilution in sterile PBS, pH 7.4) or polymorphoneutrophils or mouse peritoneal macrophages ( $1 \times 10^6 \text{ mL}^{-1}$ ) were added to the culture reaction. After 15 minutes of incubation for whole blood and 30 minutes for isolated cells, 25  $\mu\text{L}$  ( $7 \times 10^5 \text{ M}$ ) luminol (G-9382 Sigma Chemical Co. USA) or 25  $\mu\text{L}$  (0.5 mM) lucigenin were added, followed by 25  $\mu\text{L}$  (20 mg  $\text{mL}^{-1}$ ) SOZ (Sigma Chemical Co. USA) or 25  $\mu\text{L}$  (40 nM) PMA.

HBSS<sup>++</sup> alone was run as a control. The level of ROS for the compounds was monitored for 50 minutes by the Luminometer (Luminoskan RS, Labsystems, Finland). The total ROS level was recorded as total light produced and recorded during 50 minutes scan.

## 2.5 T-Cell proliferation assay

Cell proliferation was evaluated by standard thymidine incorporation assay following a method reported by Nielsen et al. (Nielsen et al., 2000). 10 mL blood was collected by vein puncture in heparin containing tube from a healthy volunteer. 50 mL sterile falcon tube was placed in safety cabinet (at sterile conditions) to this was added blood, ficoll paque and RPMI incomplete media (without fetal bovine serum) in equal volume. 5 mL ficoll paque was added in three 15 mL sterile empty centrifuge tubes and centrifuged at 400xg for 20 minutes at room temperature with no break. Peripheral blood mononuclear cells (PBMCs) appeared at the junction of two layers, the upper layer was discarded and the lower containing the PBMCs was transferred in another sterile tube, washed with RPMI incomplete media and centrifuged again at 300xg for 10 minutes at 4°C. The supernatant was discarded and re-suspended pellet in 1mL RPMI incomplete media and stored on ice. The viability of the cells was checked by trypan blue and cells were counted by hemocytometer. 50  $\mu\text{L}$  of 5% complete RPMI was added into each well of a sterile 96 well plate in sterile environment using safety cabinet followed by samples (drug and its derivatives) having the concentration between 6.25 and 100  $\mu\text{g mL}^{-1}$  and adjusting the final volume to 0.3 mL. While well 'A' contained only 5% complete RPMI to be used as control. 50  $\mu\text{L}$  PBMC cells ( $1 \times 10^6 / \text{mL}$ ) were added in suspension of 5% complete RPMI to each well except blank followed by the addition of 50  $\mu\text{L}$  PHA except negative control and blank and volume of well was made up to 0.2 mL by 5% complete RPMI. The mixture was incubated for 72 hours in CO<sub>2</sub> incubator at 37°C. After incubation 25  $\mu\text{L}$  of thymidine was added in each well except blank and the plate again incubated in CO<sub>2</sub> incubator at 37°C for 18 hours. After incubation cells were harvested using glass fiber filter and the cell harvester. In cell harvesting the plates were washed with 70% ethanol 5 times, filtered and left until they were completely dry, then dissolved in separate vials using the scintillation solution and scanned by scintillation counter.

## 2.6 Cytokine production from mononuclear cells

The monocytes were grown in 75 mm<sup>2</sup> flask in RPMI-1640 supplemented with 10% FBS until they attained 70% confluency and passaged twice a week. On reaching confluency

the cells were plated in 24-well tissue culture plates at a concentration of  $2.5 \times 10^5$  cells/mL. The cells were differentiated into macrophage like cells by using phorbol myristate acetate (PMA) at a final concentration of  $20 \text{ ng mL}^{-1}$  and incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 24 hours.

After 24 hr incubation with PMA, cells were stimulated with bacterial lipopolysaccharide (LPS)  $50 \text{ ng mL}^{-1}$  and treated with compounds (drug and its derivatives) at a concentration of  $25 \text{ }\mu\text{g mL}^{-1}$  and incubated for 4 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The supernatants were collected after 4 hrs which was then analyzed for the level of  $\text{TNF-}\alpha$ . The plate was incubated for further 18 hrs and then supernatants were collected for the analysis of  $\text{IL-1}\beta$  level. Human  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  Duo set Kits (R&D systems, Minneapolis, USA) were used for cytokine quantification according to manufacturer's instructions (Singh et al., 2005).

## 2.7 General procedure for preparation of derivatives (A6-A12)

Enoxacin and all the chemicals used were of analytical grade. Amines used for the synthesis were urea, thiourea, acetamide, thioacetamide, hydroxylamine, benzamide and phthalimide). The compounds were prepared as summarized in figure 2 and 3.

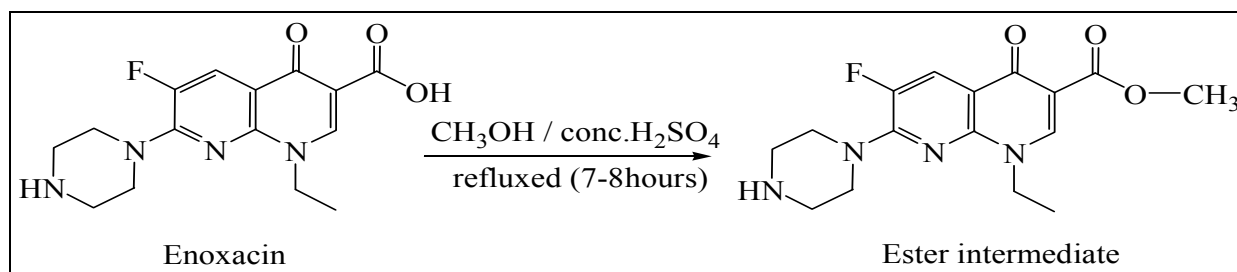


Fig. 2. Synthesis of ester intermediate

0.01 mole of enoxacin (3.21 g) was dissolved in hot methanol (80 mL) to which 1.2 mL sulphuric acid was added and the solution was refluxed for about 7-8 hours till the consumption of drug in ester formation (monitored by TLC). The solution was cooled down to room temperature and the precipitate obtained was washed with chloroform and dried for 1 hour at  $80^\circ\text{C}$  to give methyl ester of the drugs. The corresponding esters were subjected to nucleophilic attack by adding 0.01 molar methanol solutions of amines respectively with continuous stirring to generate carboxamides. While for compound **A11** and **A12**, NaOH was added in the methanolic mixture of benzamide and phthalimide and warmed for 30 mins prior to their addition in the reaction flask containing enoxacin ester intermediate. The reaction was refluxed for about 2-3 hours till completion, indicated by TLC. The volume of the reaction mixture was then reduced by rotary-evaporation. The precipitates were filtrated off, washed with chloroform and re-crystallized from methanol-chloroform (2:8) mixture till pure compounds were achieved (checked on TLC and constant melting point).



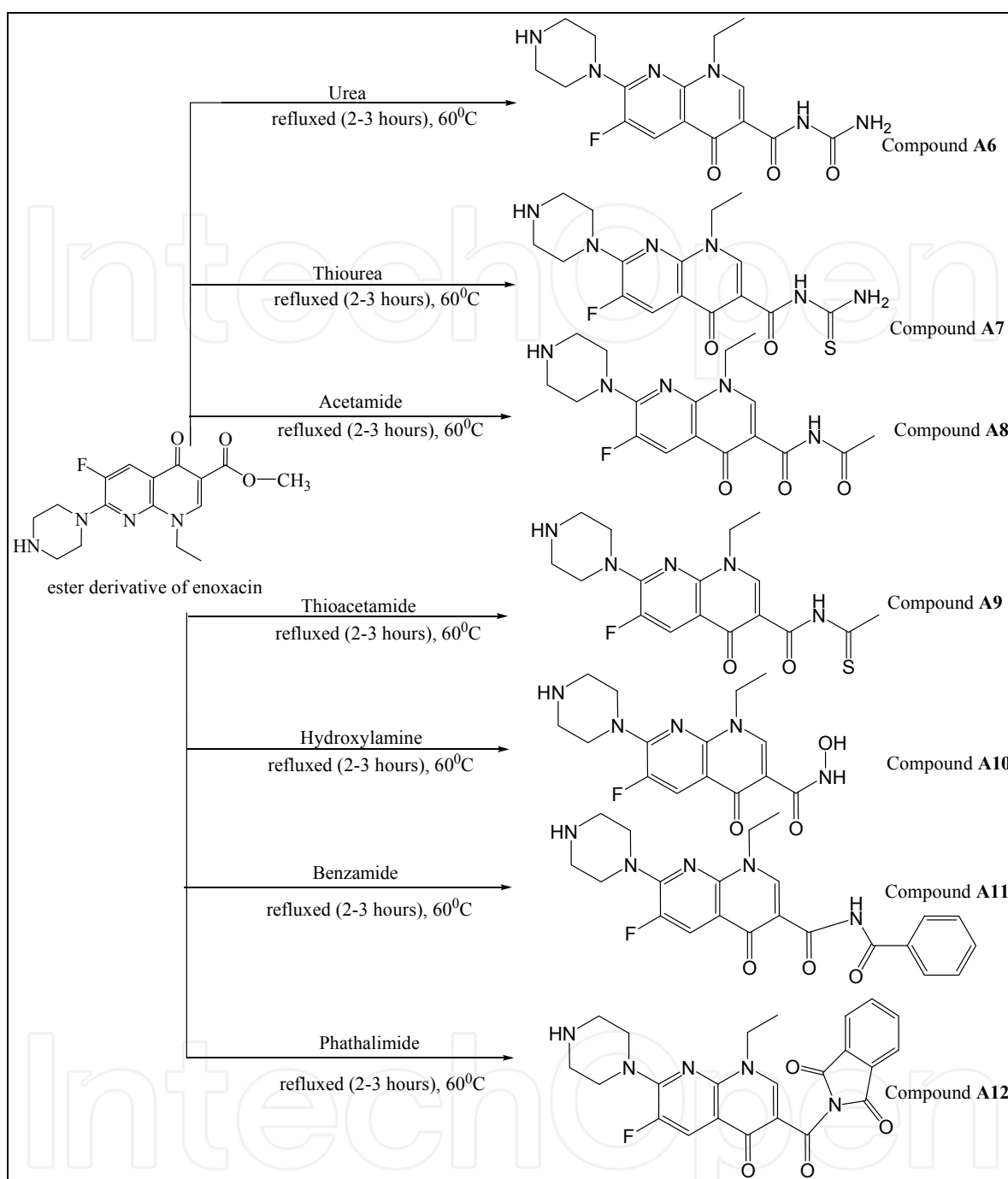


Fig. 3. Synthesis of compound A6-A12

### 3. Result and disscussion

#### 3.1 Spectral analysis

##### 3.1.1 IR studies

In the IR spectrum of enoxacin, the OH stretching vibration of carboxylic acid appeared as a broad band in the range 3500–3100  $\text{cm}^{-1}$  and interfered with the NH stretching vibration of the piperizynyl ring which absorbed in the same region at

3100  $\text{cm}^{-1}$ . Two strong carbonyl absorptions at 1690 and 1640  $\text{cm}^{-1}$  were observed due to the presence of keto carboxylic acid.

The spectra of all the compounds showed absence of acidic OH peak of enoxacin and a distinct, strong and un-obscured NH stretch was observed near 3200-3100  $\text{cm}^{-1}$  indicating that carboxylic site reacted with the selected amines forming amides. However compound **A10** showed a sharp band at 3300  $\text{cm}^{-1}$  due to the presence of hydroxyl group in their structure. While compounds **A7** and **A9** showed a medium intensity band near 2100  $\text{cm}^{-1}$  due to the presence of C=S group, additionally, IR spectra of the compounds revealed that carbonyl absorption of carboxylic group shifted towards right near 1665-1640  $\text{cm}^{-1}$  in the spectra of all the compounds which suggests the utilization of carboxylic moiety in amide formation.

### 3.1.2 $^1\text{H}$ NMR studies

Important  $^1\text{H}$  NMR signals of enoxacin were observed at chemical shifts of 1.40 (t, 3H,  $J = 7.0$  Hz,  $-\text{CH}_3$  methyl), 2.0 (s, 1H, amine), 2.62 (s, 4H, piperazine), 3.85 (s, 4H, piperazine), 4.48 (q, 2H,  $J = 7.0$  Hz,  $-\text{CH}_2-$  ethyl), 8.10, 8.95 (s, 2H, naphthyridine) and 11.0 (s, 1H, COOH). On comparing main peaks of enoxacin with its derivatives, all the signals of enoxacin were present in the  $^1\text{H}$ NMR spectra of the compounds except the acidic proton resonance at 9.8 ppm. Moreover, all these derivatives displayed resonance of naphthyridine protons down-field by (0.4-0.7 ppm). Unlike enoxacin all the derivatives showed a singlet in the region 7.9-8.0 ppm which corresponds to the absorption of sec-amide. Further, the spectra of the compounds also exhibited other signals corresponding to their respective chemical structure (mentioned in the spectral data). The signals for the aliphatic and piperazine protons were practically unchanged since they lie far from the reaction site of the drug.

### 3.1.3 Mass spectrometric studies

1. The electron impact mass spectrum (EIMS) of enoxacin showed molecular ion ( $\text{M}^+$ ) peak at ( $m/z$ , %): (320, 35). However compounds (**A6-A12**) showed a very low percentage of  $\text{M}^+$  peaks with respect to their proposed structures.

### 3.1.4 Spectral data of compounds **A6-A12**

#### **A6 1-(1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carbonyl)urea**

M.p. 257°C IR (KBr)  $\nu_{\text{max}}$ : 1655, 1625 (C=O) and 3150 (N-H)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.41 (t, 3H,  $J = 7.2$  Hz,  $-\text{CH}_3$  ethyl), 2.59 (s, 4H, piperazine), 3.84 (s, 4H, piperazine), 5.6 (s, 4H, amine), 4.45 (q, 2H,  $J = 7.0$  Hz,  $-\text{CH}_2-$  ethyl), 7.98 (s, 1H, sec. amide), 8.20 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.49$  Hz), 9.18 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (362, 10.5)  $\text{M}^+$ .

#### **A7 1-(1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carbonyl)thiourea**

M.p. 287°C IR (KBr)  $\nu_{\text{max}}$ : 1655, 1630 (C=O), 2100 (C=S), 3300 (OH), 3200 (N-H) and 2100 (C=S)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.43 (t, 3H,  $J = 7.1$  Hz,  $-\text{CH}_3$  ethyl), 2.62 (s, 4H, piperazine), 3.83 (s, 4H, piperazine), 4.2 (s, 4H, amine), 4.45 (q, 2H,  $J = 7.2$  Hz,  $-\text{CH}_2-$  ethyl), 7.95 (s, 1H, sec. amide), 8.21 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.51$  Hz), 9.19 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (378, 9.5)  $\text{M}^+$ .



**A8 N-acetyl-1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide**

M.p. 295°C IR (KBr)  $\nu_{\max}$ : 1655, 1630 (C=O) and 3140 (N-H)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.42 (t, 3H,  $J = 7.0$  Hz,  $-\text{CH}_3$  ethyl), 2.02 (s, 3H, methyl), 2.61 (s, 4H, piperazine), 3.84 (s, 4H, piperazine), 4.46 (q, 2H,  $J = 7.1$  Hz,  $-\text{CH}_2-$  ethyl), 7.85 (s, 1H, sec. amide), 8.21 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.56$  Hz), 9.19 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (361, 89.5)  $\text{M}^+$ .

**A9 N-ethanethioyl-1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide**

M.p. 237°C IR (KBr)  $\nu_{\max}$ : 1645, 1628 (C=O), 2100 (C=S), 3300 (OH), 3150 (N-H) and 2120 (C=S)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.41 (t, 3H,  $J = 7.1$  Hz,  $-\text{CH}_3$  ethyl), 2.529 (s, 3H, methyl), 2.62 (s, 4H, piperazine), 3.81 (s, 4H, piperazine), 4.44 (q, 2H,  $J = 6.9$  Hz,  $-\text{CH}_2-$  ethyl), 7.98 (s, 1H, sec. amide), 8.21 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.54$  Hz), 9.19 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (377, 5.5)  $\text{M}^+$ .

**A10 1-ethyl-6-fluoro-N-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide**

M.p. 267°C IR (KBr)  $\nu_{\max}$ : 1655, 1634 (C=O), 3300 (OH) and 3180 (N-H)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.43 (t, 3H,  $J = 7.0$  Hz,  $-\text{CH}_3$  ethyl), 2.62 (s, 4H, piperazine), 3.84 (s, 4H, piperazine), 4.45 (q, 2H,  $J = 7.0$  Hz,  $-\text{CH}_2-$  ethyl), 6.58 (s, 1H, OH), 7.97 (s, 1H, sec. amide), 8.18 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.56$  Hz), 9.09 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (335, 11)  $\text{M}^+$ .

**A11 N-benzoyl-1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxamide**

M.p. 255°C IR (KBr)  $\nu_{\max}$ : 1645, 1635 (C=O) and 3155 (N-H)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.42 (t, 3H,  $J = 7.0$  Hz,  $-\text{CH}_3$  ethyl), 2.62 (s, 4H, piperazine), 3.86 (s, 4H, piperazine), 4.43 (q, 2H,  $J = 7.0$  Hz,  $-\text{CH}_2-$  ethyl), 7.21 (m, 5H, phenylic H), 7.99 (s, 1H, sec. amide), 8.18 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.56$  Hz), 9.1 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (423, 7)  $\text{M}^+$ .

**A12 2-(1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxyl)isoindoline-1,3-dione**

M.p. 278°C IR (KBr)  $\nu_{\max}$ : 1655, 1630 (C=O) and 3150 (N-H)  $\text{cm}^{-1}$ ,  $^1\text{H}$  NMR (DMSO, 500 MHz) 1.43 (t, 3H,  $J = 7.0$  Hz,  $-\text{CH}_3$  ethyl), 2.60 (s, 4H, piperazine), 3.85 (s, 4H, piperazine), 4.45 (q, 2H,  $J = 7.0$  Hz,  $-\text{CH}_2-$  ethyl), 7.69 (s, 2H, phenylic H), 8.3 (s, 2H, phenylic H), 7.94 (s, 1H, sec. amide), 8.15 (d, 1H,  $\text{H}_5$ -naphthyridine,  $J_{\text{H,F}} = 12.56$  Hz), 9.09 (s, 1H,  $\text{H}_2$ -naphthyridine), MS ( $m/z$ , %): (449, 10)  $\text{M}^+$ .

**3.2 Antimicrobial studies****3.2.1 Antibacterial activity**

All compounds showed activities nearly similar to enoxacin against all the *Gram-positive* test strains. Generally, the 7-piperazinyl enoxacin have better *Gram-negative* than *Gram-positive*

antimicrobial potency (Chin & Neu, 1983, Paton & Reeves, 1988, Wood, 1989). Studies revealed that the mode of action of quinolones involves inhibition of essential type II bacterial topoisomerases such as DNA gyrase and topoisomerase IV (Sissi & Palumbo, 2003). Commonly DNA gyrase is more sensitive to *Gram-negative* bacteria and topoisomerase IV more sensitive to *Gram-positive* bacteria. As topoisomerase IV is the primary target of the quinolones with bulky functional group at N-4 position of piperazine ring, therefore the activities of all the compounds were found to be similar to enoxacin.

However, against all the *Gram-negative* organisms, strong improvement in the antibacterial activity of the derivatives **A11**, **A12** and **A6** was observed in comparison to enoxacin (Tables 1 and 2). The increase in activity was greatest against *Salmonella typhi*, *Pseudomonas aeruginosa* and *Escherichia coli*. The enhancement in the antibacterial activity of these compounds might be a result of better interaction with DNA gyrase. It was observed that the activity of compounds **A7-A10** decreased against all the *Gram-negative* strains. It is proposed that in terms of structure-activity relationship, the antibacterial activity profile of enoxacin derivatives against *Gram-negative* bacteria was enhanced by the phenyl attachment via amide linkage at the 3-position of the enoxacin molecule. While reaction with aliphatic amines caused a diminution in antibacterial activity of enoxacin, exception was compound **A6**.

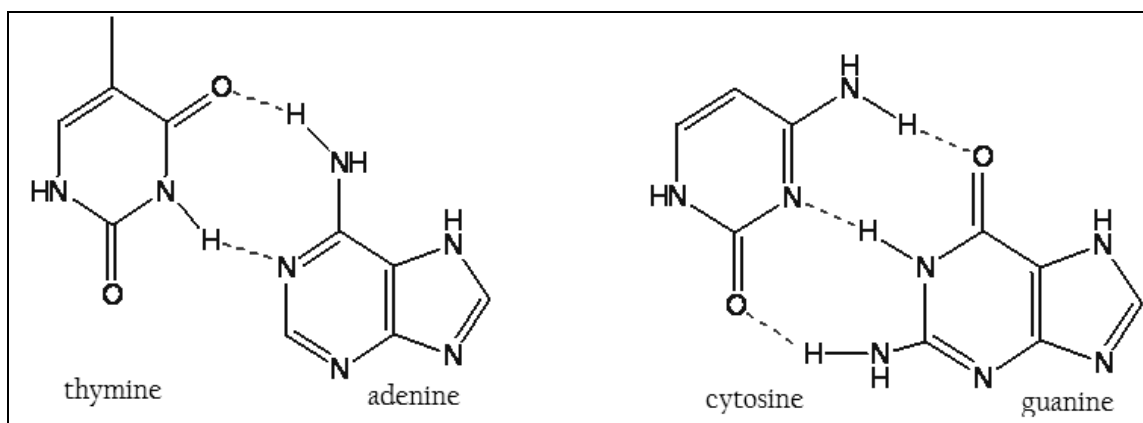


Fig. 4. Base pairing between adenine/thymine & cytosine/guanine

Previous studies showed that carboxylic group of quinolones was necessary to bind to bacterial DNA gyrase through hydrogen bonding. Looking at the structure of DNA, it is apparent that the hydrogen bonding between the two DNA strands is also through the amide group of heterocyclic bases.

Since the inhibitory action of quinolones is not simply accomplished by inhibiting the bacterial enzyme function but they also actively poison cells by trapping the two topoisomerases on DNA as drug/enzyme/DNA complexes in which double-strand DNA breaks are held together by protein. It is proposed that converting carboxylic group into amide group would help quinolone to better undergo binding with bacterial DNA due to structural complementarities.

Microorganisms	Enoxacin			A6			A7			A8		
	$\mu\text{gmL}^{-1}$			$\mu\text{gmL}^{-1}$			$\mu\text{gmL}^{-1}$			$\mu\text{gmL}^{-1}$		
	5	10	20	5	10	20	5	10	20	5	10	20
<i>S. aureus</i>	14	16	16	12	16	18	11	15	15	10	12	13
<i>Citrobacter</i>	13	14	19	12	14	18	10	11	12	9	10	10
<i>S. pneumoniae</i>	11	13	17	11	12	16	11	12	13	12	14	15
<i>S. flexneri</i>	10	12	15	12	14	18	11	11	12	10	12	13
<i>E. coli</i>	12	19	20	13	19	23	10	11	12	11	12	14
<i>S. typhi</i>	10	15	20	12	15	28	9	10	11	11	14	18
<i>P. aeruginosa</i>	13	14	14	14	16	18	11	12	13	9	10	11
<i>B. subtilis</i>	10	12	14	12	14	16	9	10	11	11	12	14
<i>K. pneumoniae</i>	12	13	16	13	15	17	10	12	14	10	11	12
<i>P. mirabilis</i>	11	12	14	11	13	16	9	11	12	10	10	12
<i>C. hofmannii</i>	10	10	12	11	13	14	9	10	11	9	10	11

Table 1.

Microorganisms	A9			A10			A11			A12		
	$\mu\text{gmL}^{-1}$			$\mu\text{gmL}^{-1}$			$\mu\text{gmL}^{-1}$			$\mu\text{gmL}^{-1}$		
	5	10	20	5	10	20	5	10	20	5	10	20
<i>S. aureus</i>	11	12	14	11	14	14	11	12	15	10	14	16
<i>Citrobacter</i>	12	12	13	14	15	17	10	12	21	10	15	22
<i>S. pneumoniae</i>	12	14	16	13	14	15	11	14	16	13	14	17
<i>S. flexneri</i>	11	12	14	11	13	14	11	12	16	11	13	17
<i>E. coli</i>	12	14	16	12	14	18	13	20	26	12	18	25
<i>S. typhi</i>	11	13	16	12	15	16	14	17	26	11	15	27
<i>P. aeruginosa</i>	10	12	13	10	11	12	10	14	20	10	11	19
<i>B. subtilis</i>	11	13	13	11	14	14	11	13	13	11	14	14
<i>K. pneumoniae</i>	11	12	13	14	15	16	11	12	18	10	15	16
<i>P. mirabilis</i>	11	12	13	10	12	12	9	12	17	10	12	16
<i>C. hofmannii</i>	9	10	11	8	9	10	9	11	11	9	9	12

Table 2.

3.2.1 Antifungal activity

Quinolone antibiotics have been shown to be potent inhibitors of DNA gyrase and have been useful antibacterial drugs in clinical practice (Marklein, 1996). Laboratory work has indicated that inhibition of DNA gyrase is not specific and activity against nonbacterial targets has been shown (Dykstra et al., 1994, Fostel et al., 1996). Nakajima et al., (Nakajima et al., 1995) have reported that quinolone augmented the activity of amphotericin B and

fluconazole against a variety of fungi in both *in vitro* and *in vivo* assays of combined antifungal activity. Based on these studies, we aimed to evaluate the antifungal activities of enoxacin and its derivatives against common fungal pathogens; *Aspergillus parasiticus*, *Saccharomyces cerevisiae*, *Candida albicans* and *Fusarium solani*. Results show that except compound **A6**, none of the tested compounds possessed clinically useful antifungal activity. Compound **A6** showed moderate inhibitory activity against *Candida albicans* (zone of inhibition = 17mm). The increased activity is probably due to some intracellular interaction of the derivative within the fungal cell; however the mechanism of the observed antifungal effect is still warranted (Sugar et al., 1997).

### 3.3 Immunomodulatory activities

Fluoroquinolones have been studied for their modulatory activity on the immune response. The best investigated agent seems to be moxifloxacin which showed immunomodulatory actions both *in vitro* and *in vivo*. However, the molecular mechanism causing the immunomodulatory effects of fluoroquinolones are still under investigation (Tauber & Nau, 2008). Recently we have reported few carboxamide analogues of enoxacin showing potentials to mediate immune response.

In order to test the immunomodulatory effect of enoxacin and its derivatives, we investigated their effect on phagocytosis, T-cell proliferation and cytokine release (particularly IL-1 $\beta$  and TNF- $\alpha$ ) by macrophages.

#### 3.3.1 Effect on phagocytes oxidative burst

Phagocytic cells on activation induce release of reactive oxygen species (oxidative burst) which is then quantified by a luminol-lucigenin enhanced chemiluminescence assay. With the luminol probe which detects the intracellular ROS using serum opsonized zymosan as activator, compounds **A11** and **A12** showed moderate inhibition on whole blood (IC<sub>50</sub> 10.5 and 14.4  $\mu\text{g mL}^{-1}$  respectively). Similar inhibitory effects were observed on the isolated neutrophils (IC<sub>50</sub> = 11.2 and 13.6  $\mu\text{g mL}^{-1}$  respectively) and on macrophages (IC<sub>50</sub>, 13.2 and 11.4  $\mu\text{g mL}^{-1}$ ). In another set of experiment lucigenin probe and PMA as activator were used as a replacement for luminol and SOZ, only compounds **A12** again demonstrated a moderate inhibition on the isolated neutrophils (12.6  $\mu\text{g mL}^{-1}$ ) respectively). Enoxacin and other compounds did not exert any significant inhibition up to the highest concentration (25  $\mu\text{g mL}^{-1}$ ) in the system tested.

#### 3.3.2 Effect on T-cell proliferation

The anti-proliferation of the test compounds was determined by measuring the inhibition of (PHA)-induced T-cell proliferation by determining radioactive thymidine incorporation. Results show (Figure 7) that compound **A11** significantly suppressed T-cell proliferation in dose dependent manner. A dose as low as 3.15  $\mu\text{g mL}^{-1}$  of compound **A11** caused approximately 50% reduction in T cell proliferation compared to control. While enoxacin and other compounds did not have any effect on T-cell proliferation up to the highest concentration (50  $\mu\text{g mL}^{-1}$ ) in the system tested.

Figure 5 shows the immunomodulatory effects of compound **A11** exhibiting prominent effect on PHA-induced T-cell proliferation. Cells were incubated with different

concentrations of the test compound in RMPI along with PHA for 72 hrs at 37°C in CO<sub>2</sub> environment. Cells were further incubated for 18 hrs after the addition of thymidine [<sup>3</sup>H] and the radioactivity count as CPM reading was recorded using scintillation counter. The effect of compound on the T-cell proliferation is compared with the control. Each plot and error bar represents readings ± SD of three repeats.

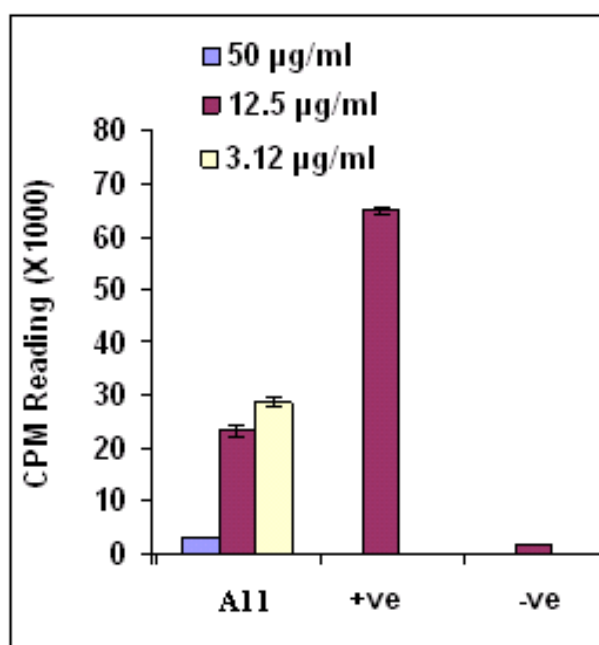


Fig. 5. Immunomodulatory effects of compound A-11

### 3.3.3 Effect on cytokine release by macrophages

The immuno-suppressive effect of enoxacin and its derivatives were tested on the release of selected cytokines including IL-1 $\beta$  and TNF- $\alpha$  by PHA-induced macrophages. Only compound **A11** at concentration of 25 $\mu$ g mL<sup>-1</sup> fairly suppressed the production of TNF- $\alpha$  showing 70% inhibition. Enoxacin and other compounds demonstrated no effect on any of the tested cytokines released from activated macrophages.

## 4. Conclusion

In conclusion we have synthesized some novel enoxacin derivatives bearing various aliphatic and aromatic substituents at C3 position via amide linkage. It was found that introduction of amide linkage with phenyl substituent at C3 position, produced noticeable enhancement in the *in vitro* activity of enoxacin particularly against Gram negative organism. In addition, unlike enoxacin, Compound **A6** also exhibited effective anti-fungal activity against *Candida albicans*. Moreover, the phagocytic function, T-cell proliferation and cytokine release was moderately affected in presence of compounds **A11** and **A12**, thus showing potentials to be anti-inflammatory. Conclusively, the test compounds showing diverse beneficial biological activities could serve as new lead molecular entities for treating various conditions such as infections, organ transplantation, cancer and for the treatment of rheumatoid arthritis and related autoimmune disorders. However, appropriate clinical trials are necessary before using the antimicrobial and immunomodulatory property of the test compounds in clinical practice.



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