Advanced Journal of Microbiology Research Vol. 2012
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

In vitro anti-bacterial activity of a novel isoquinoline derivative and its post antibacterial effects on *Pseudomonas aeruginosa*

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Accepted 15 March, 2012

The isoquinolines are of great importance to humanity because of their medicinal value and different structure. There have been many researches on the isoquinolines, but relatively few pure compounds have been investigated for their antibacterial activity. The in vitro activity of a novel synthetic antimicrobial compound 1-(4-choloro-phenyl)-6,7-dimethoxy-2-methyl-1,2,3,4,- tetrahydroisoquinoline was evaluated against Pseudomonas aeruginosa (ATCC27853) using standard methods approved by Clinical and Laboratory Standards Institute. The minimum inhibitory concentration of the compound which inhibited more than 90% growth (MIC90) of P. aeruginosa was found to be ranged from 24.0 to 6.0 □ g mł1 in different media. The ET50 (concentration which lyses 50% erythrocytes) of the compound was observed to be 450 □ g mł1. It was found that the compound down-regulated the expression of PQS, elastase and pyocyanin, the important virulence factors of P. aeruginosa. Proteomics studies revealed that the compound inhibited/down regulated the expression of PhnA and oprL proteins of P. aeruginosa which are crucial for PQS synthesis and membrane integrity. This type of compounds may provide avenue for the discovery of clinically useful antibacterial drugs.

Key words: Isoquinoline, *P. aeruginosa*, virulent factors, antimicrobial activity, proteomics.

INTRODUCTION

Pseudomonas aeruginosa is the most common gram-negative bacterium found in nosocomial infections. It is an opportunistic pathogen that primarily causes infections in immunocompromised patients of cystic fibrosis, neutropenia, iatrogenic immunosuppression or who suffer from serious burn wounds (Fagon et al., 1996; Hancock, 1998; Kiska and Gilligan. 1999). Moreover, *Pseudomonas* maintains antibiotic resistance plasmids, both R-factors and RTFs, and it is able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against *Pseudomonas*, including fluoroquinolone, gentamicin and imipenem. These antibiotics are also not effective against all strains because of the various efflux systems (Poole et al, 1993; Kohler et al, 1997), presence of μ-lac- tamase and impermeable membrane (Hancock, 1998). Therefore, it is the leading cause of nosocomial infections and hospital-acquired pneumonia (Jarvis and Martone, 1992).

Isoquinoline alkaloids are reported to have various kinds of biological activities including antibacterial acti-vity. Isoquinoline alkaloids are reported to interfere with protein kinases which are important for signal transduction pathways. In the present study *in vitro* activity and effect of the isoquinolone derivatives on the expression of virulence factors of *P. aeruginosa* were exa-mined. The expression of various virulence factors was found to be down-regulated after treatment with the compound the expression of PhnA and oprL proteins which are impor-

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tant for PQS synthesis and membrane integrity.

METHODS

Microorganisms

Four Clinical isolates of *P. aeruginosa*, obtained from MLB Medical College, Jhansi, (UP), India, were used along with the standard strain of *P. aeruginosa* (ATCC 27853).

Antimicrobial agent

The compound 1-(- 4-choloro-phenyl-) 6, 7-dimethoxy- 1,2,3,4,-tetra-hydro isoquinolone was synthesized in the laboratory (Tiwari et al, 2005) and dissolved in dimethyl sulphoxide (384.0 μ g ml^{-1}). Stock solution (96.0 $\mu g\ ml^{-1}$) was prepared by further dilution with water.

Microbroth dilution assay

Microbroth dilution assays for the evaluation of susceptibility were performed as per CLSI document (CLSI, 2006). An adjusted inoculum of the test organism was inoculated into Mueller–Hinton broth (Hi-Media) containing two fold dilutions of an initial compound solution so that each well contained approximately 1.0 X 10^5 c.f.u. ml^{-1}.

Tests were also performed in RPMI 1640 (Gibco-BRL) buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acids (MOPS; Sigma), RPMI 1640+5% glucose, RPMI 1640+10% glucose, Luria-Bertani broth and nutrient broth. The minimum concentration of compound which inhibited any visual growth of *P. aeruginosa* was considered as minimum inhibitory concentration (MIC_{90}). All the experiments were repeated thrice.

Hemolytic assay

Hemolytic assay was performed as described earlier (Yadav et al., 2005). Various concentrations of isoquinoline compound ranging from 1024.0 to 2.0 $\mu g\ ml^{-1}$ were used in the study. In comparison with gentamicin, the percent hemolysis was calculated and plotted against concentration of compound to determine the ET_{50} of compound to erythrocytes.

Growth curve

Bacteria were grown in Luria-Bertani medium in absence or presence of compound. The bacteria were treated with different concentrations of the compound ranging from 2.0 to 20 $\mu g\ ml^{-1}$. The OD600 of the cultures at different time intervals was measured.

Pyocyanin and 2-heptyl-3-hydroxy-4-quinolone (PQS) assays

Pyocyanin assays were performed as described previously (Gallagher and Manoil, 2001) . Sample of culture supernatants (4 ml) from 5 ml cultures (treated and non-treated) grown with aeration for 16 h at 37^0C from an initial inoculum at OD 600 of 0.02 were extracted with 3.0 ml of chloroform. Chloroform phase was further extracted with 0.5 ml of 0.2 N HCl and the OD_{520} of aqueous phase was measured. PQS analysis of the compound in treated and non-treated cultures was performed as described by Pesci et al. (1999).

Hemolysin assay

Blood agar plates (containing 10% (vol vol^{-1}) defibrinated sheep blood) were used to detect hemolysin activity of single colonies. The hemolytic activity was determined by measuring the zone of hemolysis formed around the single colonies after 12 h incubation at 37°C.

Study of differentially expressed proteins

P. aeruginosa was cultured with sub-MIC dose (8.0 $\mu g\ ml^{-1}$) of the compound for 10 h at 37^0C. Bacterial cultures were centrifuged at 3000 Xg for 10 min and washed with saline water. The bacteria were suspended in phosphate saline buffer (pH 7.2) and optical density at 600 nm was adjusted to 0.6 (Shimadzu-UV 1700). The uniformity of the OD was confirmed by plating the bacterial suspension on nutrient agar plates. The same volumes of bacterial cells were lysed directly into the SDS-PAGE sample buffer. The proteins obtained from different cultures were resolved by SDS-PAGE for comparison. The stained gels were scanned using a gel scanner (Vilber Lourmat) and the images were analyzed by Kodak 1D.

MALDI-TOF MS

The proteins were eluted from the gels directly and re-electrophoresed for further purification. Samples were analyzed using a MALDI- TOF (Applied Bio-systems) employing standard protocols. Peptide Mass Fingerprint of Mascot programs were used to identify the proteins from MALDI-TOF/MS spectra.

RESULTS AND DISCUSSION

Multiple drug resistance caused by a variety of mechanisms in *P. aeruginosa* has been reported to increase morbidity and mortality (Carmeli et al., 1999; Harris et al., 1999; Levin et al., 1999; Livermore, 2002). The trend of increasing resistance in this gram-negative organism is even more disturbing, as most of the current new experimental antibacterial efforts are directed toward gram-positive pathogens (Flamm et al., 2004).

The compound 1-(-4-choloro-phenyl-) 6,7-dimethoxy-1,2,3,4,- tetrahydro isoquinolone was evaluated against four clinical isolates and one standard strain of *P. aeruginosa* for its antibacterial activity. MIC_{90} for the compound was observed in a range of 6.0 to 24.0 $\mu g\ ml^{-1}$ in various media (Table 1). Variations in MIC_{90} were observed due to medium effects and lowest MIC_{90} 6.0 $\mu g\ ml^{-1}$ was observed in LB broth. The hemolytic assay showed that the fifty percent erythrocytes were lysed by the compound at concentration of 450 $\mu g\ ml^{-1}$ which was less than that of gentamicin (Figure 1). The growth curves of the bacteria in the presence or absence of compound showed that the exponential as well as stationary phases were shifted downward with increasing concentration of the antimicrobial compound (Figure 2). Colony sizes of the treated cultures were also observed to be moderate as compared with the cultures treated with gentamicin.

PQS and the PQS-controlled virulence factor, pyocyanin were observed to be decreased in the compound treated cultures. The comparative TLC densitometry profiles show significantly less secreted amount of PQS in the cultures treated with sub-MIC doses of compound

Table 1. Effect of different test media on *in vitro* activity of 1-(4-choloro-phenyl)-6,7-dimethoxy-2-methyl-1,2,3,4,-tetrahydroisoquinoline against four clinical isolates of *P. aeruginosa.*

Isolate	MIC_{90} (μg ml^{-1})					
	Mueller–Hinton broth	RPMI 1640	RPMI 1640 +2% glucose	RPMI 1640 +5% glucose	Luria Bertani broth	Nutrient broth
156/02	24.0	24.0	24.0	24.0	12.0	12.0
197/03	24.0	24.0	24.0	24.0	12.0	12.0
1245/05	24.0	24.0	24.0	ND	24.0	12.0
845/06	12.0	12.0	24.0	24.0	12.0	12.0
ATCC 7853	12.0	12.0	24.0	24.0	6.0	12.0
Gentamycin	2.2	2.2	2.5	2.5	2.0	2.2

ND, Not Determined.

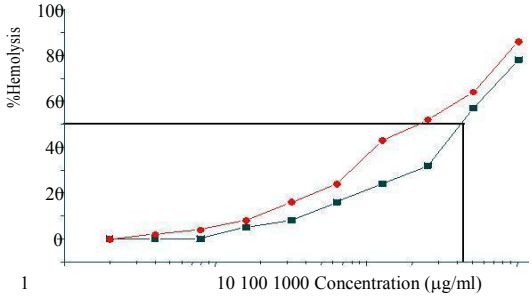

Figure 1. Toxicity of compound analyzed by hemolytic assay. The zero and 100% hemolysis were determined by using buffer and Triton X 100 respectively in control sets. The values of hemolysis determination were repeated three times and graph plotted against concentration of compound ( ) and standard drug gentamicin ( ) on log10 scale.

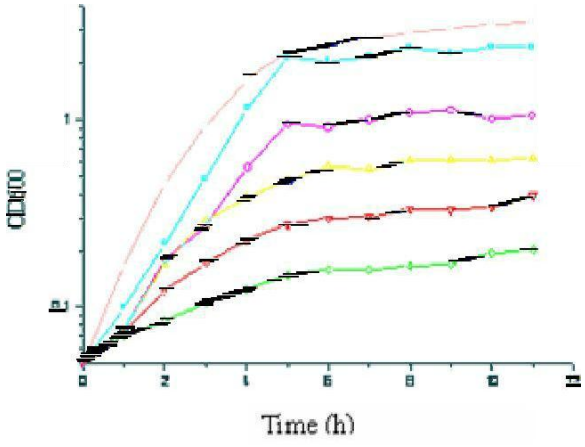

Figure 2. Bacterial strains were grown in Luria-Bertani medium in absence of ( ) or in presence of {( ) 2.0; (o), 4.0; ( ) 8.0; ( ) 10.0 and ( ) 20.0 μmg ml-1)} of the compound. Growth curve determinations were repeated three times and graph shows result from one typical experiment.

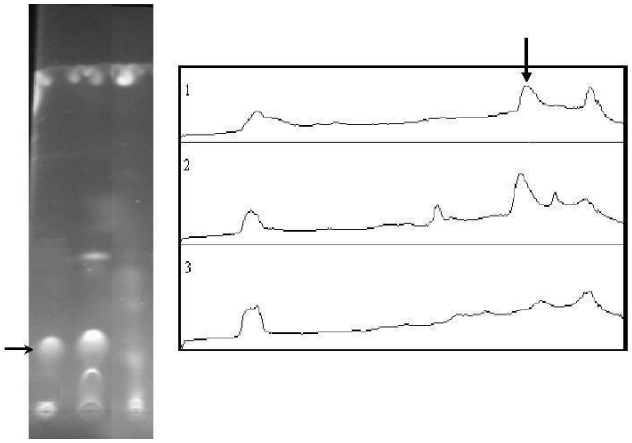

Figure 3. Effect of 1-(4- chlorophenyl)- 6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline on PQS production. (A): PQS production by *P. aeruginosa* strains treated with the isoquinoline derivative. PQS samples extracted from 24-h cultures were analyzed by TLC. Lanes 1: Standard of PQS; Lane 2: Control cultures; Lane 3: Cultures treated with the compound. The arrowhead indicates the position of PQS in each lane. (B): Comparative profiles of TLC by using Kodak 1D software.

compound (Figure 3). Pyocyanin production in compound treated cultures was found to 12 time less than normal culture which makes a significant difference ($p < 0.001$). Hemolytic activity was also found to be reduced significantly in the cultures treated with the compound. Clear zones were observed around the *P. aeruginosa* colonies formed due to secretion of hemolysin in untreated cultures. No lysis zones were observed around the colonies of bacteria treated with sub-MIC doses of compound (Figure 4).

Post antibacterial effects were examined by comparesson of protein profiles of bacteria in the presence or absence of compound. *P. aeruginosa* was cultured in the presence of sub- MIC dose (8.0 μg ml^{-1}) of the compound for 10 h at 37 ^{0}C. The four proteins of 18.0, 35.4, 54.4 and 96.0 kDa were observed to be inhibited by the presence

Table 2. Identification of *P. aeruginosa* proteins using SDS-PAGE and MALDI-TOF.

Spot No	MOWSE score	Protein MW (kDa)	Theoretical tryptic peptides	Peptide matches	Accession number	Protein name
1	79	54.55	39	8	D35114	PhnA
2	75	35.426	25	7	G83533	Hypothetical Protein
3	73	17.959	10	5	S58217	OprL

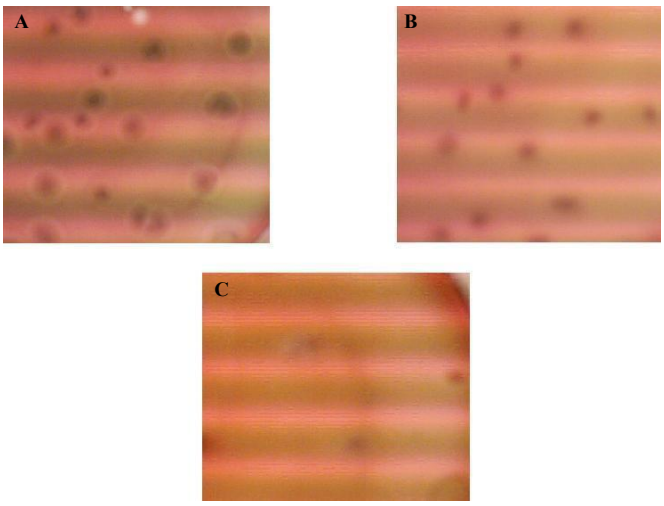

Figure 4. *P. aeruginosa* growing on blood agar plates, showing hemolysin activity in (A) non-treated, (B) treated with compound and (C) Treated with Gentamycin.

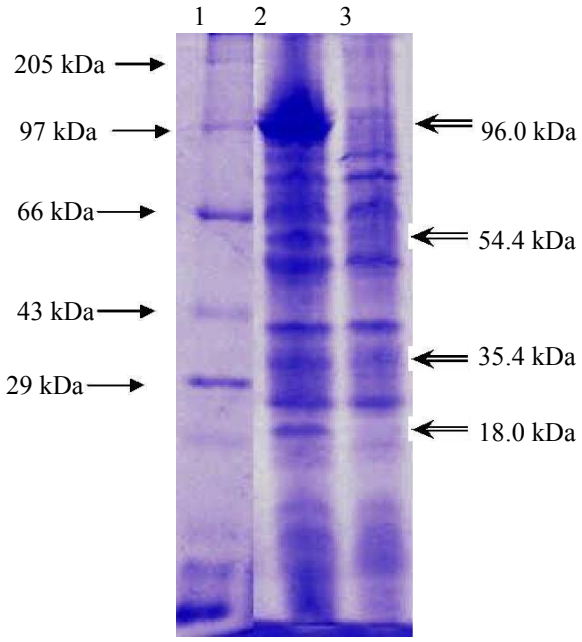

Figure 5. Protein profile of the *P. aeruginosa* treated with 1-(4-chloro-phenyl)-6,7- dimethoxy- 1,2,3,4-tetrahydro-isoquinoline. (A) Lane 1: Molecular weight marker; Lane 2: *P. aeruginosa* without any treatment; Lane 3: Protein profile of *P. aeruginosa* treated with the compound.

of compound in the cultures (Figure 5). The four down-regulated proteins having clear resolution on SDS-PAGE were re-electrophorsed and analyzed by MALDI-TOF. Proteins having MOWSE score above 70 were considered to be significant (Table1). Proteins of 18.0, 35.4 and 54.4 kDa showed similarity with oprL, hypothetical and PhnA, respectively (Table 2). The down-regulated protein phnA is an important protein for the synthesis of PQS (Pesci et al., 1999). PhnA and PhnB presumably synthesize the anthranilate precursor of PQS from chorismate (D'Argenio et al., 2002; Reimmann et al., 1997) . The oprL is an outer membrane protein, which plays a key role in the maintenance of outer membrane integrity and cell morphology in gram-negative bacteria (Essar et al., 1990) . The tol-oprL region in *P. aeruginosa* is reported to be involved in pyocyanin uptake and required for cell viability (Essar et al., 1990).

How the compound down-regulates the synthesis of *phn* A, *opr*L and other two genes products are not known. But the compound 1-(-4-choloro-phenyl-) 6,7-dimethoxy-1,2,3,4,- tetrahydro isoquinolone may be used as an avenue for discovery of novel useful synthetic antimicrobial compound.

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