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Antibacterial and synergy of clavine alkaloid lysergol and its derivatives against nalidixic acid resistant *Escherichia coli*

Synergistic antibacterial potentials of clavine alkaloids

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Abstract

Antibacterial activity of lysergol (**1**) and its semi-synthetic derivatives (**2-14**) and their synergy with the conventional antibiotic nalidixic acid (NA) against nalidixic acid sensitive (NASEC) and nalidixic acid resistant (NAREC) strains of *Escherichia coli* was evaluated. Lysergol (**1**) and derivatives (**2**-**14**) did not possess antibacterial activity of their own, but in combination, they

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significantly reduced the minimum inhibitory concentration (MIC) of NA. All the derivatives showed two to eight folds reduction in the MIC of NA against NAREC and NASEC. Further, lysergol (**1**) and its derivatives **10** and **11** brought down eight fold reductions in the MIC of tetracycline (TET) against multidrug resistant clinical isolate of *E. coli* (MDREC). Treatment of these strains with the combinations of antibiotics and lysergol and its derivatives **10** and **11** (at reduced concentrations) significantly decreased the viability of cells. In an another observation, lysergol and its derivatives **10** and **11** inhibited ATP dependent efflux pumps, which was evident by ATPase inhibition and down regulation of multidrug ABC transporter ATP binding protein (yohI) gene. These results may be of great help in antibacterial drug development from a very common, inexpensive and non toxic natural product.

Keywords: Lysergol; acyl/aryl analog; bioenhancer; multidrug resistant, efflux pumps.

Introduction

The consumption of antibiotics by man is increasing at an alarming rate. Out of the total antibiotics we use, 20%-50% of it is unnecessary depending on the class of antibiotic [1]. This may be due to (i) reduced absorption in the gut membrane when taken orally (ii) restrictive uptake by the target microbe or (iii) operation of efflux pump leading to indiscriminate extrusion of the antibiotics. So the major amounts of the antibiotics we apply are wasted and only a minor percentage is being targeted to the infective microbes. Further, the unutilized antibiotic amount remains as a load in the body and environment acting as a selection pressure facilitating emergence of drug resistance in parasites and their predominance, ultimately leading to failure of antibiotics against resistant infections [2, 3].

One of the ways, which has been feasible to reduce drug dosage, has been synergism between two therapeutic agents. However, if both have the antibiotic property, still the problem of continued selection pressure on microbes is likely to continue. So, we thought of searching only those molecules, which by them are not microbicidal but when present with an antibiotic, enhances its activity and availability (bioenhancers) [2, 4]. In this way these molecules (bioenhancers) will not exert any selection pressure for mutants to emerge resistant against them and on the other hand could reduce the dosage of antibiotics so that their ill effects are minimized and the resistance development process will be substantially delayed ultimately leading to enhanced life-span of the novel and existing antibiotics. Such antibiotics facilitators should have novel properties like non-toxic to human, animal or plants should be effective at a very low concentration in a combination, should be easy to formulate and most importantly enhance uptake/absorption and activity of the antibiotics. This can lead in developing judicious and strategic concentrations of antibiotics with specific bioenhancers to improve availability of the drug right up to the target for effectively controlling the infectious organisms [2, 4].

Drug resistant *E. coli* has become the most common cause of many life-threatening diseases such as infection of the bloodstream. Recent data from the U.S. National Healthcare Safety Network indicate that gram-negative bacteria are responsible for more than 30% of hospital-acquired infections of which *E. coli* predominate in cases of urinary tract infections (45%) [5]. The present study primarily deals with drug resistance reversal potential of lysergol (**1**) and its semi-synthetic derivatives with the conventional antibiotic, nalidixic acid against

NASEC and NAREC strains of *E. coli*. The most active combinations were validated by checkerboard and time kill assays against NASEC and NAREC as well as with another antibiotic tetracycline against MDREC. Further efflux pump inhibition assay, ATPase inhibitory and real time expression analysis were carried out to find the possible mode of action of lysergol and its derivatives.

Material and methods

General experimental procedures

The NMR spectra were recorded on Avance 300 MHz spectrometer (Bruker). The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks. MS were recorded on hyphenated LC-PDA-MS (Prominence LC and mass MS-2010EV, Shimadzu). The compounds were first vizualized on TLC plates (silica gel $60F_{254}$, Merck) under UV illumination at 254 and 365 nm and then sprayed with Dragon Droff's reagent.

Antibacterial agents

The conventional antibiotics nalidixic acid and tetracycline were purchased from Sigma (purity ≥98%). Lysergol was isolated and identified from the seeds of *I. muricata* as described in the previous reports [6, 7]. Further, acyl and aryl derivatives of lysergol were prepared according to the procedure given below. The purities of isolated and semi-synthetic derivatives were ≥95 % (HPLC).

Chemical derivatization of lysergol (1)

Lysergol (50 mg) was dissolved in pyridine (1.5 mL) and to this solution respective acyl / aryl chloride were separately added in a 1:1.5 ratio using 4-dimethylaminopyridine (DMAP) as catalyst (Figure 1). The airtight reaction mixture was kept overnight at room temperature and the progress of the reaction was monitored by TLC. After completion of the reaction, ice cold water was added and the mixture was extracted with chloroform (3 x 20 mL). The pooled chloroform extract was washed with water until neutral and dried over anhydrous Na_2SO_4 and evaporated under vacuum. This chloroform extract was further purified by flash chromatography over Silica gel to yield the respective derivatives in 75–90% yields. All the derivatives (**2**-**14**) were characterized on the basis of their ^{1}H, ^{13}C NMR and mass spectroscopic data (see supplementary file).

Bacterial strains

E. coli strains, nalidixic acid resistant DH5α (MTCC 1652) NAREC was procured from MTCC, IMTEC Chandigarh, India, while nalidixic acid sensitive strain CA8000 (NASEC) was donated to CIMAP repository by Dr. Sushil Kumar, Ex Director CIMAP Lucknow, India [8]. The well characterized multi drug resistant clinical isolate-KG4 (MDREC) was gifted by Dr. Mastan Singh and Dr M.K. Gupta, King George Medical University, Lucknow [9].

Media

Standard Mueller-Hinton agar and broth (MHA and MHB, Hi-Media, Mumbai, India) were used as bacterial culture media. MHB was used for susceptibility testing. Colony counts were determined using MHA plates.

Susceptibility and Synergy testing

The test compounds were diluted into final concentrations of 1000 to 1.9 µg/mL and tested against DH5α, CA8000, and MDREC strains of *E. coli*. The MIC values were determined by 2-fold serial dilution broth assay [10, 11] with starting inoculums of 5×10^5 cfu/ml, incubated at 37°C for 24 h and detected from the observatory data as per CLSI guidelines [12] using nalidixic acid as positive control. They were determined in duplicate, with concentrations ranging from 0.39 to 200 µg/mL of nalidixic acid.

The combination studies were performed at concentrations ranging from 0.39 to 200 µg/mL of nalidixic acid in combination with 10 µg/mL of each test compound [3, 13]. The microtitre plates (Genaxy) were inoculated with 10 µL of diluted overnight grown culture of the test organism with a titre equivalent to 0.5 McFarland standards. The inoculated microtitre plates were then incubated at 37°C for 24 h. The final bacterial inoculum in each well was $5x10^5$ CFU/mL. The results were recorded in terms of reduction. The reported MIC values (µg/mL) and fold reductions are the mean of 3 experiments in replicate. Results were recorded in the term of fold reduction.

In addition, synergy studies of most active combinations were also performed by broth checkerboard method [14]. Cation-adjusted Mueller-Hinton broth (150µL) was added to each well of the 96-well plate. The last two columns of wells served as controls for *E. coli* growth and plate sterility. The final concentrations ranged from 1.56 to 800 µg/mL for tetracycline and from 1.25 to 160 µg/mL for test compounds. Thus, each of the 80 wells had a unique combination of antibiotics and test compounds. The final bacterial inoculum in each well was $5x10^5$ CFU/ml except the negative control. The plates were incubated at 37°C for 24 hours. The MIC was recorded as the last dilution without any turbidity as per CLSI guidelines.

Time kill studies

The time kill study of nalidixic acid and tetracycline alone and in combination with lysergol (**1**) and its derivatives **10** and **11** against *E.coli* strains was conducted at MIC, 2MIC and 4MIC concentrations using a method described previously by Eliopoulus and Moellering [15]. Each analysis was done in triplicate with a control without test sample. Time kill curves were derived by plotting $\log_{10}$ CFU/ml against time (h). Time kill kinetics was also studied in combinations of antibiotics and test compounds at the reduced concentrations at which maximum synergy was observed.

Ethidium bromide efflux studies

The fluorometric determination of ethidium bromide efflux was performed as described previously [16]. Bacterial (MDREC) culture was grown to reach optical density (OD) of 0.6 at 600 nm. The cells were collected by centrifugation and washed with PBS. The suspension (0.3 OD) was exposed to 5 µg/mL ethidium bromide for 60 min at 25°C in the presence of lysergol (**1**) and its derivatives **10** and **11** at 10 µg/mL. The cells were harvested by centrifugation and resuspended in fresh buffer. Loss of fluorescence was recorded for 30 min at 1 min intervals at an excitation and emission wavelength of 530 nm and 585 nm respectively using spectrofluorometer (FLUO star omega, BMG Labtech Germany).

ATPase inhibitory activity of phytomolecules

The bacterial membrane protein was isolated by the method described earlier [17]. ATPase assay was carried out using quantichromTM ATPase Assay Kit (BioAssay Systems, USA) and ATPase activity was estimated by measuring liberated inorganic phosphate (Pi) spectrophotometrically (18).

qRT– PCR analysis of the ATP dependent efflux pump protein of E. coli

The transcriptional profile of the multidrug ABC transporter ATP binding protein (yohI) gene was analyzed in treated and non treated cells of MDREC by the method described earlier [19]. Cells were grown to mid-log phase in the presence of sub-inhibitory concentration (1/4 MIC) of tetracycline, lysergol (**1**) and its derivatives **10** and **11** alone and in combination. The real-time quantification of the RNA templates was analyzed by SYBR GreenER qPCR super mix (Invitrogen, USA) using 7900HT fast real time PCR system (Applied Biosystems, USA). Observations were recorded in terms of LogRQ after normalization of indigenous gene (gapdh) expression.

Results and discussion

Although, few antibiotics for gram-positive bacteria are in the drug discovery pipeline, but there are none for the gram-negative bacteria. It has been found that efflux pumps are the main cause of development of multi drug resistance (MDR); hence there is urgent need for the search of efflux pump inhibitors and synergistic combinations of drugs, which can reverse the phenomenon of MDR [20, 21].

The present study was aimed to isolate and identify plant molecules, which can reverse the phenomenon of multidrug resistance specifically in human pathogenic class of gram-negative bacteria. Clavine alkaloid lysergol was isolated and identified from the seeds of *I. muricata* as described in the previous reports [6, 7].

Antibacterial activity evaluation of the clavine alkaloid lysergol against one nalidixic acid sensitive (NASEC) and one nalidixic acid resistant (NAREC) strains of *E. coli*, showed that lysergol do not posses antibacterial activity (MIC 1000 µg/mL). But when 10 µg/mL of alkaloid was tested separately in combination with nalidixic acid, it showed significant synergistic activity. It remarkably reduced the MIC of nalidixic acid by eight folds against the NAREC and four folds against the NASEC. This potential synergistic effect of lysergol prompted us to prepare some new derivatives for their enhanced activity. A total of thirteen semi synthetic acyl and aryl derivatives (**2**-**14**) of lysergol (**1**) were prepared as described in the material and method section. All the derivatives (**2**-**14**) except **2** were found to be new and characterized on the basis of their ^{1}H, ^{13}C NMR and mass spectroscopic data. The purities of isolated clavine alkaloid lysergol (**1**) and semi-synthetic derivatives (**2-14**) were ≥95 % (HPLC). Further, these derivatives when evaluated for their synergistic potential against the NASEC and NAREC strains of *E. coli*, it was observed that two aryl derivatives **10** and **11** reduced the MIC of nalidixic acid by eight folds against the NASEC, which is actually two times to that of lysergol. On comparing these observations with the known drug resistance reversal agent, reserpine [22, 23], it was observed that clavine alkaloids lysergol and most of the derivatives exhibited two to four times better activity than the reserpine (Table 1).

The effective concentrations of lysergol (**1**) and its derivatives **10** and **11** were determined through checkerboard assay and found to be 10 µg/mL (Table 2; see suppl. file). Further, these observations were validated by using another antibiotic tetracycline against MDREC and similar types of data were collected wherein reduction in the MIC of tetracycline up to 8 folds was recorded (Table 2). Similar type of results were also reported earlier where it was found that some alkaloids did not possessed antibacterial activity but in combinations they were able to reduce the dose of partner drugs many folds [13, 22].

The treatment of NASEC, NAREC with NA at MIC, 2MIC and 4MIC concentrations reduced the viability of *E. coli* significantly (Figure 2A, 3A). However, the bactericidal activity of NA was achieved at very low concentration when tested in combination with lysergol (**1**) and its derivatives **10** and **11** (Figure 2B, 3B). Similar type of data was also recorded when another antibiotic tetracycline (TET) was tested against multidrug resistant strain MDREC of *E.coli* (Figure 4A, 4B). Our observations are in accordance with earlier reports wherein bactericidal activity was achieved at sub-inhibitory concentrations when tested in combinations [23, 24].

In order to understand possible mechanism of action of lysergol (**1**) and its two derivatives **10** and **11**, they were subjected to fluorescence based ethidium bromide efflux assay using multidrug resistant strain MDREC of *E.coli*. As shown in Figure 5, significant decrease in fluorescence was observed in non treated control cells. While in presence of lysergol (**1**) and its two derivatives **10** and **11**, the loss of fluorescence was significantly reduced, reflecting a strong interference with ethidium bromide efflux by these compounds. Accumulation and efflux of

ethidium bromide are good indicators of the involvement of efflux pumps in the resistance mechanism, particularly in Gram negative bacteria such as *E. coli* [25, 26].

Further, to understand whether these compounds interfere with ATP dependent efflux pump, they were evaluated for ATPase inhibitory activity in MDREC. It was found that lysergol (**1**) and its derivatives **10** and **11** significantly inhibited ATPase activity (Figure 6) in terms of liberated inorganic phosphate (Pi) indicating involvement of these compounds in the inhibition of ATP dependent efflux pumps. Similar studies have been reported earlier for understanding the mechanism of action of known drugs such as reserpine, ouabain and phenothiazine [27].

The involvement of these compounds in the inhibition of ATP dependent efflux pump was further validated by studying the expression of multidrug ABC transporter ATP binding protein (yohI) gene upon the treatment of MDREC by lysergol (**1**) and its derivatives **10** and **11**. As evident from the Figure 7, up regulation of this gene was observed when the cell were treated with tetracycline (1/4 MIC). While, this gene was found to be significantly down regulated upon the treatment of MDREC with lysergol (**1**) and its derivatives **10** and **11**. Similarly, expression of yojI gene was found to be down regulated in presence of the combinations of tetracycline and test compounds at the concentrations where maximum synergy was achieved (Figure 7). Tetracycline is known to induce higher expression of different efflux pump genes [19, 28] and inhibition/modulation of this activity by the compounds reported in this study indicates towards their potential as drug resistance reversal agents.

Conclusions

From the above results, it may be concluded that lysergol (**1**) and its derivatives **10** and **11** are the novel efflux pump inhibitors being reported for the first time. Inhibition of the efflux pump yojI by these efflux pump inhibitors can be useful in: (i) lowering the dose of antibiotics; (ii) reducing the drug resistance development frequency; and (iii) increasing the efficacy of antibiotics against multidrug resistant *E.coli* strains. These results may be of great help in the development of inexpensive and dose economic antibacterial drug formulations from a very common and widely distributed herb, *I. muricata*.

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Figures Captions

Figure 1. Chemical derivatization of lysergol (1)

Figure 2: Time–kill curves of NASEC showing the dose dependent bactericidal effect of (A) nalidixic acid; (B) nalidixic acid in combination with lysergol and its derivatives Der-10 and Der-11.

Figure 3. Time–kill curves of NAREC showing the dose dependent bactericidal effect of (A) nalidixic acid; (B) nalidixic acid in combination with lysergol and its derivatives Der-10 and Der-11.

Figure 4. Time–kill curves of MDREC showing the dose dependent bactericidal effect of (A) Tetracycline: (B) Tetracycline in combination with lysergol and its derivatives Der-10 and Der-11.

Figure 5. Inhibition of ethidium bromide efflux by lysergol and its derivatives Der-10 and Der-11 in MDREC .

Figure 6. Inhibition of ATPase by lysergol and its derivatives Der-10 and Der-11 in MDREC .

Figure 7. Expression level of yojI mRNA in MDREC upon the induction by tetracycline alone and lysergol and its derivatives Der-10 and Der-11 alone and in combinations.

Table 1. MIC of lysergol/derivatives (2-14) alone and in combination with Nalidixic acid

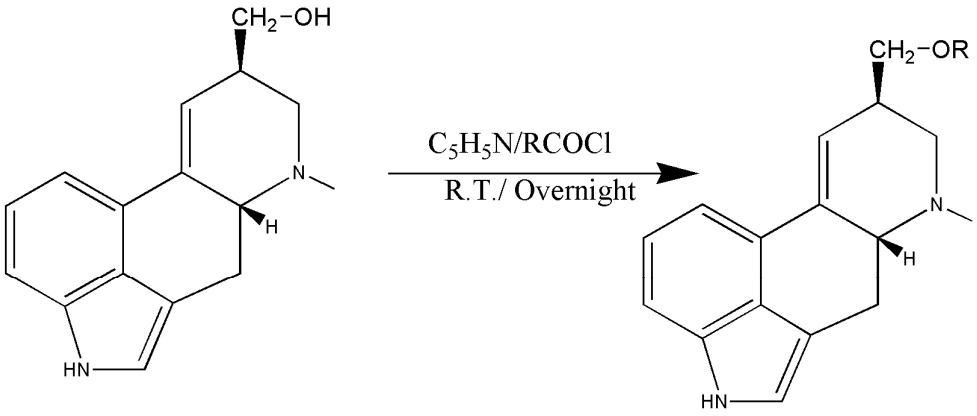
Compounds	**MIC of compounds alone against *E. coli* (µg/mL)**		**MIC of Nalidixic acid in combination with compounds (10 µg/mL)**			
	CA8000	**DH5α**	**CA8000**	**Fold reduction**	**DH5α**	**Fold reduction**
Nalidixic acid	6.25	100		-	-	-
Lys-**1**	1000	1000	1.56	4	12.5	8
Der-**2**	1000	1000	1.56	4	25	4
Der-**3**	1000	1000	3.125	2	50	2
Der-**4**	1000	1000	1.56	4	25	4
Der-**5**	1000	1000	1.56	4	12.5	8
Der-**6**	1000	1000	1.56	4	25	4
Der-**7**	1000	1000	1.56	4	25	4
Der-**8**	1000	1000	1.56	4	50	2
Der-**9**	1000	1000	1.56	4	25	4
Der-**10**	1000	1000	0.78	8	12.8	8
Der-**11**	1000	1000	0.78	8	12.5	8

Der-**12**	1000	1000	3.125	2	50	2
Der-**13**	1000	1000	3.125	2	50	2
Der-**14**	1000	1000	1.56	4	25	4
Reserpine* (RES)	500	500	3.125	2	50	2

*Known drug resistance reversal agent

Table 2. Reduction in the minimum inhibitory concentrations of antibiotics in combination with plant compounds against drug sensitive and resistant strains of *E.coli* through checker board assay.

Plant compounds (μg/mL)	**MIC of NA with/without plant compounds against**		**MIC of tetracycline with/without plant compounds against**
	NASEC	**NAREC**	**MDREC**
Lys-**1** (160)	1.56/6.25	12.5/100	100/800
Lys-**1** (10)	1.56/6.25	12.5/100	100/800
Lys-**1** (1.25)	3.125/6.25	50/100	200/800
Der-**10** (160)	0.78/6.25	12.5/100	100/800
Der-**10** (10)	0.78/6.25	12.5/100	100/800
Der-**10** (1.25)	3.125/6.25	50/100	400/800
Der.-11(160)	0.78/6.25	12.5/100	100/800
Der-**11**(10)	0.78/6.25	12.5/100	100/800
Der-**11**(1.25)	3.125/6.25	25/100	200/800
Reserpine (RES, 160)	3.125/6.25	50/100	400/800
Reserpine (10)	3.125/6.25	50/100	400/800
Reserpine (1.25)	6.25/6.25	100/100	800/800



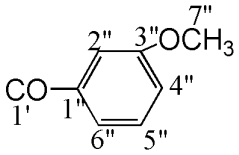
Lysergol (**1**) acyl / aryl derivatives of lysergol (**2-14**)

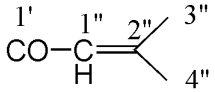
2. R= $CO\overset{1'}{C}H_3$ 1"

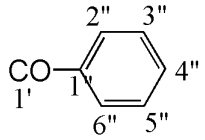
3. R= $\overset{1'}{C}O(\overset{1"\text{-}12"}{C}H_2)_{12}\overset{13"}{C}H_3$

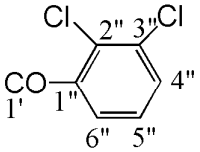
4. R= $\overset{1'}{C}O(\overset{1"\text{-}10"}{C}H_2)_{10}\overset{13"}{C}H_3$

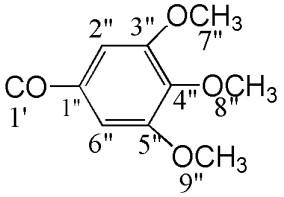
5. R= $\overset{1'}{C}O(\overset{1"\text{-}14"}{C}H_2)_{14}\overset{13"}{C}H_3$

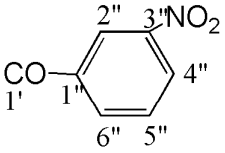
6. R= 

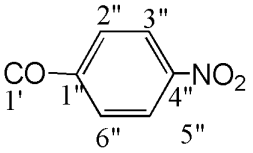
7. R= 

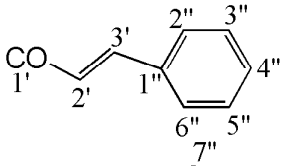
8. R= 

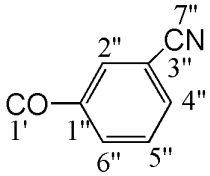
9. R= 

10. R= 

11. R= 

12. R= 

13. R= 

14. R= 

1=Lys (lysergol), **2** = Der-2 (17-*O*-acetyllysergol), **3** = Der-3 (17-*O*-meristoyllysergol), **4** = Der-4 (17-*O*-lauroyllysergol) **5** = Der-5 (17-*O*-pamitoyllysergol), **6** = Der-6 (17-*O*-m-anisoyllysergol), **7** = Der-7 (17-*O*-3",3"-dimethylacryloyllysergol), **8** = Der-8 (17-*O*-benzoyllysergol), **9** = Der-9 (17-*O*-2",3"-dichlorobenzoyllysergol), **10** = Der-10 (17-*O*-3",4",5"-trimethoxybenzoyllysergol), **11** = Der-11 (17-*O*-3"-nitrobenzoyllysergol), **12** = Der-12 (17-*O*-4"-nitrobenzoyllysergol), **13** = Der-13 (17-*O*-cinnamoyllysergol), **14** = Der-14 (17-*O*-3"-cyanobenzoyllysergol)