

Antibacterial, antifungal, anti-oxidant, anti-inflammatory and anti-hypertensive activities of N-chloropyrazinamide

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ABSTRACT

N-chloropyrazinamide was prepared by the chlorination of pyrazinamide using trichloroisocyanuric acid. The experimental results ascertained that the tested N-chloropyrazinamide showed good antimicrobial activities when compared to standard drug. It was found that N-chloropyrazinamide at different concentrations exhibited significant dose dependent anti-oxidant, anti-inflammatory and anti-hypertensive activity.

Keywords: antibacterial activity; antifungal activity; anti-oxidant activity; anti-inflammatory; anti-hypertensive activity; N-chloropyrazinamide

1. INTRODUCTION

Great attention has been paid for curing diseases caused by microorganisms. Many infectious pathogenic microorganisms develop resistance against the prevailing drugs, and this situation has necessitated a search for new source of compounds. Functionalized nitrogen and oxygen containing heterocycles play a predominant role in medicinal chemistry and they have been intensively used as scaffolds for drug development. The rapid assemble of molecular diversity is an important goal of synthetic organic chemistry and is one of the key paradigms of modern drug discovery.

Pyrazinamide is one of the most effective anti-tuberculous drugs. Pyrazine and pyrazinamide analogs exhibited potent antibacterial activity, e.g. pyrazinoic acid esters¹, pyrazine thiocarboxamide² and ring substituted pyrazinylchalcones³. Pyrazine carboxamide derivatives have been reported to possess diverse pharmacological activities including antimicrobial activity, fungicidal activity, herbicidal activity, anti-oxidant activity and anti-algal activity⁴⁻¹¹. Although the pyrazine ring system has proved to be an interesting class in heterocyclic chemistry; it has received little attention in the literature. Some of its derivatives

are important as anticancer agents with low toxicity¹², anti-inflammatory¹³, blood platelet aggregation inhibitors, bone metabolism improvers, adenosine antagonists and controlling herbicides¹⁴. They also show antifungal and antiparasitic activities¹⁵. In addition, they are used as disperse dyes and as fluorescents.

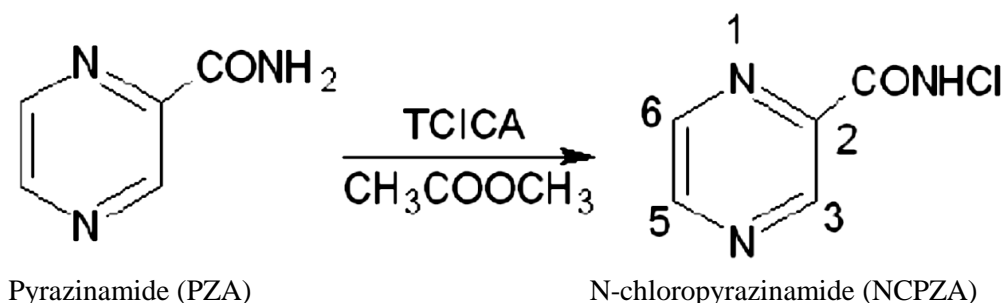
2. MATERIALS AND METHODS

All the reagents and solvents used were purchased from Merck, India and used without further purification.

3. EXPERIMENTAL

3. 1. Preparation of N-chloropyrazinamide

A simple procedure was developed for the preparation of N-chloropyrazinamide (NCPZA). In a 100 mL round-bottom flask, 0.6894 g (5.605 mmol) of pyrazinamide (PZA) was dissolved in methyl acetate. Trichloroisocyanuric acid, 0.478 g (2.057 mmol) was added and a precipitate of cyanuric acid formed after 20 min. After stirring for 4 h, the mixture was vacuum filtered and the solid was washed with methylene chloride. The solvent was removed from the filtrate using a rotary evaporator. The solid obtained was washed with diethyl ether to give high purity of N-chloropyrazinamide¹⁶. Yield 88 %; M.P. 135 °C, Scheme 1.



Scheme 1. Reaction for the preparation N-chloropyrazinamide.

3. 2. Antibacterial activity

Various concentrations of N-chloropyrazinamide viz., 100, 250 and 500 µg are tested for antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella aerogenes* and *Pseudomonas aeruginosa* by disc diffusion technique using Nystatin as a standard antibiotic drug. The nutrient agar prepared by the usual method, was inoculated aseptically with 0.5 ml of overnight subculture of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella aerogenes* and *Pseudomonas aeruginosa* in separate conical flasks at 30 °C and mixed well by gentle shaking. About 25 ml of the contents of the flasks were poured and evenly spread in petridish (90 mm in diameter) and allowed to set for 2 h. Muller Hinton agar (beef infusion solids 4.0 g, starch 1.5 g, casein hydrolysate 17.5 g, agar 15.0 g, final pH 7.4 ±0.2 at 37 °C) was used for antimicrobial assay. The assay plates were prepared by spread

plate technique with appropriate pathogen inoculums ($\sim 10^4$ CFU). Using a sterile cork borer, a 7 mm well was made and filled with 0.05 ml (50 $\mu\text{g}/\text{ml}$ of solution of sample in DMSO). The plates were incubated at 37 °C for 24 h and the control was also maintained with 0.05 ml of DMSO in similar manner and the zones of inhibition of the bacterial growth were measured in millimeter.

3. 3. Antifungal activity

Aspergillus niger and *Mucor* were used for testing antifungal activity by disc diffusion method. The culture was maintained on Sabouraud dextrose agar (SDA) slants. SDA medium was inoculated with 72 h old 0.5 ml suspension of fungal spores in a separate flask. About 25 ml of the inoculated medium was evenly spread in a sterilized petridish and allowed to set for 2 h. The well (4 mm in diameter) were punched in petridish and loaded with 0.05 ml (10 $\mu\text{g}/\text{ml}$) of solution of sample in DMSO. The plates were incubated at 30 °C for 4-8 h after the completion of incubation period, the zones of inhibition of growth in the form of diameter in mm was measured. Along the test solution in each petridish on well was filled up with solvent which acts as control.

3. 4. *In vitro* anti-inflammatory activity of NCPZA by hyaluronidase inhibition assay

The assay was performed according to Ling *et al*¹⁸ and Sigma Protocol. The assay medium consisting of 3 - 5U hyaluronidase (from Sigma - Aldrich, Bangalore) in 100 μl 20 mM sodium phosphate buffer pH 7.0 with 77 mM sodium chloride, 0.01 % BSA was preincubated with different concentrations of the test compound (in dimethyl sulphoxide) for 15 min at 37 °C. The assay was commenced by adding 100 μl hyaluronic acid (from Sigma - Aldrich, Bangalore; 0.03 % in 300 mM sodium phosphate, pH 5.35) to the incubation mixture and incubated for a further 45 min at 37 °C. The undigested hyaluronic acid was precipitated with 1 ml acid albumin solution made up of 0.1 % bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid (pH 3.75).

After standing at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm. The absorbance in absence of enzyme was used as the reference value for maximum inhibition. The inhibitory activity of test compound was calculated as the percentage ratio of the absorbance in the presence of test compound versus absorbance in the absence of enzyme. The enzyme activity was checked by control experiment run simultaneously, in which the enzyme was preincubated with 5 μl DMSO instead, and followed by the assay procedures described above. Compound was tested in a range of 0.1 μg - 1.0 μg in the reaction mixture. Indomethacin (Indo) was used as reference standard.

3. 5. *In vitro* anti-oxidant activity of NCPZA by free radical scavenging activity

The anti-oxidant activity was evaluated using free radical scavenging activity¹⁹ by DPPH method. Butylated hydroxyl anisole (BHA) was used as a standard anti-oxidant. Different concentrations (10 μg , 50 μg and 100 μg) of NCPZA in dimethyl sulphoxide and butylated hydroxy anisole (BHA) were taken in different test tubes. The volume was adjusted to 500 μl by adding methanol. Five millilitres of 0.1 mM methanolic solution of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was added to these tubes and shaken vigorously. A control without NCPZA, but with an equivalent amount of methanol was maintained. The tubes were allowed to stand at room temperature for 20 min. The absorbance of the samples was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free

radical scavenging activity. Radical scavenging activity was calculated using the following formula:

$$\% \text{ free radical scavenging activity} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

3. 6. *In vitro* anti-hypertensive activity of NCPZA

NCPZA was tested at three concentrations *viz.*, 1 µg, 5 µg and 10 µg, dissolved in assay buffer (10 mM HEPES buffer containing 0.3 M NaCl and 10 µM zinc sulphate) containing 20 µl kidney cortex plasma membranes (ACE enzyme source) and 1 mM Hippuryl-His-Leu as substrate. The compounds were incubated with the enzyme for 10 min at 37 °C. Then substrate was added and incubated for 45 min at 37 °C. The reaction was terminated by the addition of 1 M HCl. The yellow colour was developed by the addition of 100 µl of pyridine and 50 µl of benzene sulphonyl chloride. The yellow colour that formed is measured at 410 nm in an ELISA Plate Reader (iMARK, BIORAD)²⁰. Compounds with an inhibitory potential, block the substrate availability to the enzyme and thereby cause enzyme inhibition leading to no formation of yellow colour. The inhibition is represented in the form of percentage over control. Captopril, a known ACE inhibitor was tested in this assay as a standard compound.

4. RESULTS AND DISCUSSION

4. 1. *In vitro* antibacterial activity of NCPZA

A) *Staphylococcus aureus*: Against *Staphylococcus aureus*, NCPZA has shown to possess a maximum inhibition of 30 mm diameter at 500 µg. The minimum inhibition zone exhibited is 18 mm diameter for 100 µg. The appreciable inhibition zone noticed is 22 mm diameter 250 µg. The maximum zone of inhibition is exhibited at an optimum concentration of 500 µg against the standard inhibition of 35 mm diameter (Table 1).

B) *Escherichia coli*: Against *Escherichia coli*, NCPZA has shown a minimum inhibition zone of 20 mm diameter at 100 µg. The appreciable activity has observed at 250 µg as 30 mm diameter zone of inhibition. The other concentration of NCPZA *viz.*, 500 µg has shown 42 mm diameter zone. Hence the optimum level of antibacterial effect of NCPZA for *E.coli* is 42 mm against the standard Ciprofloxacin for which the inhibition zone is 38 mm diameter (Table 1).

C) *Klebsiella aerogenes*: Against *Klebsiella aerogenes*, NCPZA has revealed a minimum inhibition zone of 20 mm diameter at 100 µg. The appreciable inhibition zone is 18 mm diameter for 250 µg and the maximum inhibition is observed 22 mm diameter at 500 µg. Hence the optimum level of antibacterial effect of NCPZA for *K.aerogenes* is 22 mm against the standard inhibition of 30 mm diameter (Table 1).

D) *Pseudomonas aeruginosa*: Against *Pseudomonas aeruginosa*, NCPZA has shown a minimum inhibition zone of 15 mm diameter at 100 µg concentration and a maximum inhibition zone of 22 mm diameter for 500 µg concentration. The other concentration of NCPZA *viz.*, 250 µg has shown 18 mm diameter of inhibition (Table 1).

Table 1. Antibacterial activity of N-chloropyrazinamide.

S. No.	Bacterium	Zone of inhibition (mm)				
		Concentration of NCPZA (μg)			Solvent control	Standard
		100	250	500		
1.	Staphylococcus aureus (NCIM 2079)	18	22	30	-	35
2.	Escherichia coli (NCIM 2065)	20	30	42	-	38
3.	Klebsiella aerogenes (NCIM 2098)	20	20	28	-	30
4.	Pseudomonas aeruginosa (NCIM 2036)	15	18	22	-	35

Standard: Ciprofloxacin 5 $\mu\text{g}/\text{disc}$; Solvent: DMSO

4. 2. *In vitro* antifungal activities of NCPZA

A) *Aspergillus niger*: Against *Aspergillus niger*, NCPZA has shown 18 mm minimum inhibition of growth at 100 μg concentration. The moderate zone of inhibition, 20 mm has observed at 250 μg and the maximum zone of inhibition growth, 35 mm has found at 500 μg which is equivalent to the standard Nystatin fungicide at 100 units/disc. Hence it is found that NCPZA sufficiently inhibits the growth of *A. niger* (Table 2).

B) *Mucor*: The 50 % zone of inhibition growth is observed at 100 μg concentration of NCPZA and 68 % inhibition is shown at 250 μg . The total inhibition zone at 500 μg is 40 mm which can be compared with the commercial antifungal agent Nystatin (32 mm) at 100 units/disc (Table 2).

Table 2. Antifungal activity of N-chloropyrazinamide.

S. No.	Fungi	Zone of inhibition (mm)				
		Concentration of NCPZA (μg)			Solvent control	Standard
		100	250	500		
1.	Aspergillus Niger (NCIM 105)	18	20	35	-	35
2.	Mucor (NCIM 108)	16	22	40	-	32

Standard: Nystatin 100 units/disc; Solvent: DMSO.

4. 3. *In vitro* anti-inflammatory activity of NCPZA

The hyaluronidase inhibition activity has been carried out to evaluate the anti-inflammatory potential of NCPZA. The potency of NCPZA is compared with the standard indomethacin. Different concentrations of NCPZA *viz.*, 10, 50, 100 μg shows dose dependent effect of 2.87 %, 4.81 % and 7.69 % inhibition respectively where as the standard indomethacin at 10 μg concentration exhibits 75.93 % inhibition but at 50 μg and 100 μg

exhibits much higher activity *i.e.*, beyond the measurable range. Thus, the compound NCPZA exhibits mild to moderate activity compared to the standard indomethacin (Table 3).

Table 3. Percentage inhibition of hyaluronidase enzyme by the samples.

Sample	Test concentration (μg)	O.D at 600 nm	% inhibition
		A_{Max}	1.080
NCPZA	10	0.031	2.87
	50	0.052	4.81
	100	0.079	7.69
Indomethacin	10	0.820	75.93
	50	1.256	*
	100	1.340	*

* Beyond measurable range: Much higher activity.

4. 4. *In vitro* anti-oxidant activity of NCPZA

NCPZA is evaluated for anti-oxidant activity using *in vitro* free radical scavenging activity by DPPH method. In this method, various concentrations of NCPZA in dimethyl sulphoxide are used as a test drug whereas butylated hydroxy anisole (BHA) is used as standard anti-oxidant. The extract exhibits a dose dependent free radical scavenging activity.

Anti-oxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and thus prevent disease. The anti-oxidant activity of NCPZA at all the concentrations exhibits moderate effect. DPPH is usually used as a reagent to evaluate free radical scavenging activity of anti-oxidant. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. NCPZA is able to reduce the stable free radical to the yellow coloured diphenyl picryl hydrazine.

Table 4. Percentage free radical scavenging activity.

Sample	Test concentration (μg)	O.D at 517 nm	% activity
Control	-	0.799	-
NCPZA	10	0.785	1.75
	50	0.774	3.13
	100	0.745	6.76
Indomethacin	10	0.614	23.15
	50	0.412	48.44
	100	0.236	70.46

The potential decrease in the concentration of DPPH radical due to the scavenging ability of NCPZA at different concentration *viz.*, 10 μg , 50 μg and 100 μg is found to be 1.75

%, 3.13 % and 6.76 % respectively. With this method, it is possible to determine the antiradical power of an anti-oxidant compound by measuring the decrease in the absorbance of DPPH at 517 nm. A colour change from purple to yellow indicates that the absorbance decreases when the DPPH is scavenged by an anti-oxidant through donation of hydrogen to form stable DPPH molecule. Table 4 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of NCPZA and standard.

4. 5. *In vitro* anti-hypertensive activity of NCPZA

NCPZA does not show a significant inhibition of Angiotensin converting enzyme at the tested concentrations. Captopril, a standard ACE inhibitor, displays 50 % inhibition at its IC₅₀ concentration (Table 5).

Table 5. Inhibitory profile of NCPZA.

NCPZA	% inhibition
10 µg	6.3
5 µg	2.5
1 µg	8.0
Captopril (15 nm)	55.0

5. CONCLUSION

The antibacterial activity of NCPZA reveals that different levels of inhibition of growth as well as different levels of optimum concentration of the compound for the chosen bacterial species. *E. coli* has shown the maximum level of inhibition of growth of 42 mm at optimum concentration at 500 µg. *Staphylococcus aureus* and *Klebsiella aerogenes* have exhibited the maximum level of inhibition of growth 30 mm and 28 mm at optimum concentration of 500 µg. *Pseudomonas aeruginosa* has shown the maximum level of inhibition of growth 22 mm at an optimum concentration of 500 µg. Thus, NCPZA as antibacterial agent is comparable with commercial antibiotic drug, Ciprofloxacin.

The antifungal activity of NCPZA against the chosen fungi namely *Aspergillus niger* and *Mucor* showed a very good inhibition of growth at optimum concentration. *Mucor* has found to possess 40 mm zone of inhibition at 500 µg concentration against the standard Nystatin and *Aspergillus niger* has shown 35 mm growth of inhibition against the standard. Thus, NCPZA an antifungal agent is comparable with commercial antifungal agent Nystatin.

The anti-inflammatory activity of NCPZA against the standard indomethacin exhibits mild to moderate activity. There is a significant decrease in the concentration of DPPH radical due to the scavenging ability of NCPZA and standard. NCPZA is screened for anti-hypertensive activity against the standard captopriol. There is no significant inhibition of Angiotensin converting enzyme at different concentrations.

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