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In vitro Antibacterial Activity of 2, 2-Diamino-1-Azavinyl Aminoamide

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A new compound 2,2-diamino-1-azavinyl aminoamide was synthesized by the reaction of hydrazine hydrate and urea. It was screened against eleven bacteria for its antibacterial activity. At dose 30 μg disc^{-1} tested compound shows diameter of zone of inhibition within the range 12-24 mm against all of the tested bacteria which was found to be larger range as compared to kanamycin (27-36 mm) at the same dose. Minimum inhibitory concentration was obtained against *Shigella dysenteriae*, *Salmonella typhi* and *Sarcina lutea* with in the range of 8-16 μg ml^{-1}. The brine shrimp lethality of the compound was found to be 8.12 μg ml^{-1}. The tested compound possesses a pronounced cytotoxic effect as observed from mortality rate of brine shrimp nouplii but the compound (10 mg kg^{-1}) had no major toxic effect on hematological parameters in mice. In conclusion, the tested compound, 2,2-diamino-1-azavinyl aminoamide may be considered as a new one having potential antibacterial activity with less toxic effect on hematological parameters.

Key words: Antibacterial activity, 2, 2-Diamino-1-azavinyl aminoamide hematology

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Introduction

The compounds containing the semicarbazone functional group (-C-NH-N=C<) possess antineoplastic and antiviral activities (Blanj *et al*., 1968; Patel *et al*., 1993; Brooth *et al*., 1971). Semicarbazones are typically excellent coordination agents for transition elements such as iron, cobalt, nickel, copper, zinc and manganese (French and Blanz, 1966 and Michaud *et al*., 1968) which are present in biological systems. This property enables semicarbazones to chelate the iron, which is a cofactor in the ribonucleotide diphosphate reductase enzyme system. It limits the availability of deoxyribonucleotides and inhibits the synthesis of DNA (Agrawal *et al*., 1970; Moore *et al*., 1970 and 1971).

In this research paper, 2,2-diamino-1-azavinyl aminoamide, a new compound containing the semicarbazone functional group has been synthesized for the first time. Antibacterial activity of this compound has been studied. Cytotoxic characteristics and effect of this compound on hematological parameters in mice have also been evaluated.

Materials and Methods

Synthesis of 2,2-diamino-1-azavinyl aminoamide: Urea (24 gm) and hydrazine hydrate (10 gm) were dissolved in 25 ml distilled water and refluxed for 8 hour. On cooling to 0 °C 2,2-diamino-1-azavinyl aminoamide (reactions are shown in Fig. 1) crystallized out. This compound was purified by repeatedly washing with alcohol followed by dissolving in water and recrystallized by alcohol.

Kanamycin was purchased from BDH. All other chemicals were of reagent grade. Tested bacteria were collected from the Department of Pharmacy, University of Dhaka, Bangladesh.

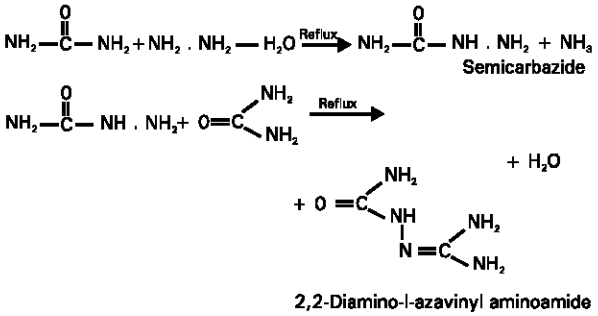

Fig.1: Synthesis of 2,2-diamino-1-azavinyl aminoamide

Antibacterial screening: Eleven bacteria (four gram positive and seven gram negative) were selected for this study. Nutrient agar was used as bacteriological media. Antibacterial potency of 2,2-diamino-1-azavinyl aminoamide was measured against all the test microorganisms according to the standard disc diffusion method (Beur *et al*., 1966) where air dried sterile Whatmann filter paper discs (6 mm diameter) with centers of at least 24 mm apart were deposited on nutrient agar plates using aseptic technique. Bacterial inoculum containing approximately, 10^4-10^6 colony forming units (CFU) per ml were spread on the surface of nutrient agar. The test compound at doses of (30, 50 and 100 μg disc^{-1}) were added into three discs. The fourth disc was supplemented with reference drug, kanamycin at dose 30 μg disc^{-1} serving as a positive control. The plates were incubated immediately at 37 °C for 14-19 hrs. Activity was determined by measuring the diameter of zones (mm) showing complete inhibition. Growth of inhibition was calculated with reference to positive control.

Minimum inhibitory concentration (MIC): The minimum inhibitory concentration of 2,2-diamino-1-azavinyl aminoamide was determined against all of the tested organisms by serial dilution technique (Jawetz *et al*., 1980). The media used in this respect were nutrient broth media. Decreasing concentration of the test compound was prepared in serial two-fold dilution using the stock solution (1.024 mg ml^{-1}). Bacterial suspension (10 μl) containing 10^7 cells ml^{-1} was inoculated into all of the tubes. After incubation for 24 hrs at 37 °C the test tube with no visible growth of the microorganism was taken to represent the MIC of test sample in μg ml^{-1}.

Brine shrimp lethality bioassay: Twelve vials were taken (two vials for each concentration) for this study. Seawater (5 ml) was given to each of the vials. The test compound at doses of 5, 10, 20, 40 and 80 μg ml^{-1} were transferred to 10 vials and two vials were used as control. With the help of pasteur pipette 10 living shrimps were inoculated into each of the vials. After 24 hours, vials were observed and numbers of survival nouplii in each vial were counted (Atta-ur-Rahman *et al*., 1999 and Meye *et al*., 1982).

LD_{50} determination: To get the LD_{50} dose of 2,2-diamino-1-azavinyl aminoamide, similar experiment was performed with Swiss Albino mice as described by Litehifield and Wilcoxon (1949). The drug solution in water was administered in different dose (i.p.) in male mice. LD_{50} was evaluated by recording mortality upto 24 hrs.

Hematological studies: For this study three groups of Swiss Albino mice (n=4) were taken. Two groups of mice were treated with 2,2-diamino-1-azavinyl aminoamide at doses 10 and 20 mg kg^{-1} i.p. for 10 consecutive days and third group of mice was considered as control. Blood was collected from individual mice for experiment on day 12 of treatment. Total counts of white blood cells (WBC) and red blood cells (RBC) as well as hemoglobin (Hb) content were determined by standard method (Rusia and Sood, 1988) using cell diluting fluids and a hemocytometer. The differential count was carried out with Wright stain.

Statistical analysis: The student–t test (Ghosh, 1984) was used for the statistical analysis of the results. P values <0.05 were considered as significant.

Results

Characterization of 2,2-diamino-1-azavinyl aminoamide: Melting point of 2,2-diamino-1-azavinyl aminoamide was found to be 195-198 °C. The elemental analysis was done with Perken Elmer 2400 Series II CHNS/O analyzer (USA). From elemental analysis, values found C: 20.21; N: 59.61; H: 5.61% as against the calculated C: 20.51; N: 59.82; H: 5.9%. Infrared spectra were recorded in KBr matrix on Parkin-Elmer Spectrophotometer in the region 4000-400 cm^{-1}. IR spectrum of the test compound exhibited peaks at 3400 and 3200 cm^{-1} indicated the presence of primary and secondary amines. Characteristic carbonyl group (>C=O) showed absorption band at 1500 cm^{-1}. Mass spectra was measured using chemical ionization (CI)/ Methane process. Base peak of the test compound was 117 (Fig. 2) which supports the molecular structure of 2,2-diamino-1-azavinyl aminoamide (NH_2-CO-NH.N=C(NH_2)$_2$

Antibacterial activity: Antibacterial activity of 2,2-diamino-1-azavinyl aminoamide was measured using doses 30, 50 and 100 μg disc^{-1}. All the gram positive and gram negative bacteria showed remarkable sensitivity to the test compound at all doses used, which were compared with standard antibacterial agent kanamycin (30 μg disc^{-1}) (Table 1). The test compound at dose (30 μg disc^{-1}) showed the lowest diameter of zone of inhibition (12 mm) against *Shigella dysenteriae* where as 28 mm diameter was observed with kanamycin at the same dose. The diameter of zone of inhibition with kanamycin (at dose 30 μg disc^{-1}) against *Bacillus subtilis, Bacillus megatarium, Sarcina lutea* and *Salmonella typhi* were found to be 29, 36, 33 and 29 mm respectively, where as the test compound (30 μg disc^{-1}) showed only 19 mm against these bacteria. The tested compound (30 μg disc^{-1}) showed the diameter of zone of inhibition 21, 24, 20, 21 and 20 mm against *Bacillus cereus, Escherichia coli, Shigella sonni,*

Table 1: Antibacterial activity of 2,2-diamino-1-azavinyl aminoamide

	Diameter of zone of inhibition (mm) after 24hr of incubation			
	2,2-Diamino-1-azavinyl aminoamide			Kanamycin
Test bacteria	30 μg disc^{-1}	50 μg disc^{-1}	100 μg disc^{-1}	30 μg disc^{-1}
Gram positive				
Bacillus cereus	21	25	30	28
Bacillus subtilis	19	21	30	29
Bacillus megaterium	19	28	33	36
Staphylococcus aureus	21	30	32	27
Gram negative				
Escherichia Coli	24	28	33	29
Shigella sonnei	20	26	32	31
Shigella shiga	21	31	33	28
Shigella boydii	20	26	33	30
Shigella dysenteriae	12	15	24	28
Sarcina lutea	19	30	33	33
Salmonella typhi	19	30	33	29

Table 2: Minimum inhibitory concentration of 2,2-diamino-1-azavinyl aminoamide against gram positive and gram negative bacteria

Test bacteria	Minimum inhibitory concentration of 2,2-diamino-1-azavinyl aminoamide (μg ml^{-1})									
Gram positive										
Bacteria	512	256	128	64	32	16	8	4	2	1
Bacillus subtilis	-	-	-	+	+	+	+	+	+	+
Bacillus cereus	-	-	-	-	+	+	+	+	+	+
Bacillus megaterium	-	-	-	+	+	+	+	+	+	+
Staphylococcus aureus	-	-	-	+	+	+	+	+	+	+
Gram negative										
Shigella dysenteriae	-	-	-	-	-	-	-	+	+	+
Shigella boydii	-	-	-	-	+	+	+	+	+	+
Shigella sonnei	-	-	-	-	-	+	+	+	+	+
Escherichia Coli	-	-	-	-	-	+	+	+	+	+
Shigella shiga	-	-	-	-	-	+	+	+	+	+
Sarcina lutea	-	-	-	-	-	-	+	+	+	+
Salmonella typhi	-	-	-	-	-	-	+	+	+	+

(+) = Growth, (-) = No growth

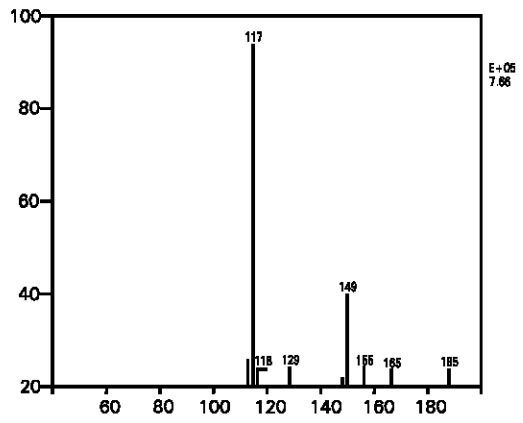

Fig. 2: Mass spectrum of the compound 2,2-diamino-1-azavinyl aminoamide

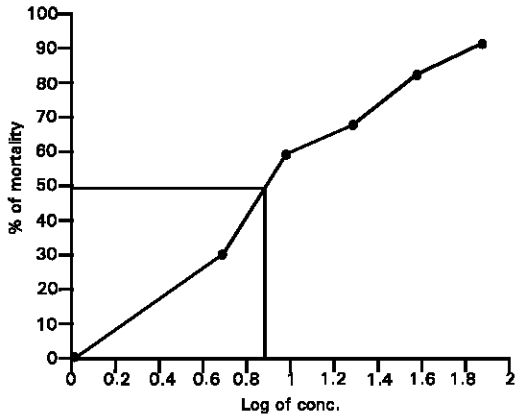

Fig. 3: Effect of 2,2-diamino-1-azavinyl aminoamide on brine shrimp mortality
Conc = Concentration of 2,2-diamino-l-azavinyl aminoamide

Shigella shiga, Shigella boydie respectively. These diameters of zones were found to be lower than those obtained with kanamycin (30 μg disc^{-1}).

Minimum inhibitory concentration: Minimum inhibitory concentration of 2,2-diamino-1-azavinyl aminoamide was found to be 8 μg ml^{-1} against *Shigella dysenteriae* (10 ^{7}cells ml^{-})^{1} [Table 2] and it was found to be 16 μg ml^{-1} against *Sarcina lutea* and *Salmonella typhi* (10^{7} cells ml^{-1}) and 32 μg ml^{-1} against *Shigella sonnei* and *Escherichia coli, Shigella shiga*.

Minimum inhibitory concentration of the test compound was found to be 64 μg ml^{-1} against *Bacillus cereus* and *Shigella boydii* and it was 128 μg ml^{-1} against *Staphylococcus aureus, Bacillus subtilis* and *Bacillus megaterium*.

Brine shrimp lethality bioassay: To find out the cytotoxic effect of 2,2-diamino-1-azavinyl aminoamide, median lethal concentration of brine shrimp (LC_{50}) was measured and it was found to be 8.12 μg ml^{-1} (Fig 3). A plot of percent mortality versus log concentration

Table 3: Effect of 2,2-diamino-1-azavinyl aminoamide on hematological parameters in mice

Parameters	Normal mice (control)	2,2-Diamino-1-azavinyl aminoamide (10 mg kg^{-1})	2,2-Diamino-1-azavinyl aminoamide (20 mg kg^{-1})
Hb (gm dl^{-1})	14.50 ± 0.54	13.33 ± 0.25	10.50 ± 0.28**
RBC ($\times 10^9$ ml^{-1})	8.33 ± 0.45	6.60 ± 0.98	5.20 ± 0.34**
WBC ($\times 10^6$ ml^{-1})	7.00 ± 0.27	9.00 ± 1.60	10.67 ± 1.00**
Lymphocyte (%)	70.00 ± 1.80	52.00 ± 3.00*	50.00 ± 2.00**
Neutrophil (%)	28.00 ± 2.70	46.00 ± 2.05**	47.50 ± 2.50**
Monocyte (%)	2.00 ± 0.54	2.00 ± 0.28	2.00 ± 0.29

Results are Mean ± SEM *: $P < 0.02$, **: $P < 0.001$

produced an approximate linear correlation between them (Fig. 3).

LD_{50} determination: Mice treated with 2,2-diamino-1-azavinyl aminoamide (150 mg kg^{-1} i.p.) did not show any toxic side effects regarding body weight of mice.

Hematological studies: At dose of 10 mg kg^{-1} i.p. show no significant change in hemoglobin, RBC and WBC content in mice blood (Table 3). Lymphocyte and neutrophil percent were found to be changed to some extent. At dose 20 mg kg^{-1} i.p. all of the hematological parameters were found to be altered significantly from the normal values.

Discussion

Study of a new compound as medicine the use of screening techniques, which can indicate its potential pharmaceutical action, is an absolute requirement. The primary bioassay screenings such as antimicrobial assays, brine shrimp lethality assay have been studied accordingly. Antibacterial activity of 2,2-diamino-1-azavinyl aminoamide was measured against some gram positive and gram negative bacteria, which are responsible for large number of human, animal and plants diseases. The test compound shows higher antibacterial activity than the known drug kanamycin. At dose 30 μg $disc^{-1}$ tested compound shows diameter of zone of inhibition within the range 12-24 mm against all of the bacteria, which was found to be larger range as compared to kanamycin (27-36 mm) at the same dose. Minimum inhibitory concentration was obtained against *Shigella dysenteriae*, *Salmonella typhi* and *Sarcina lutea*. Low value of median lethal concentration (LC_{50}) indicates the cytotoxic effect of the tested compound. There was a positive correlation between brine shrimp lethality and cytotoxicity of the compound. In this bioassay the mortality rate of brine shrimp was found to be increased with the increase of concentration of the tested compound. The test compound had no major toxic effect on hematological parameters at low dose. Considering all the above points, the compound 2,2-diamino-1-azavinyl aminoamide may be considered as a new one having potential antibacterial activity with less toxic effect on hematological parameters. The results suggest further extensive work on the test compound *in vivo*.

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