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In Vitro Inhibitory Activities of Urea Analogues on Bacterial Urease

Pan Sup Chang*, Byungse Suh, Nancy A. Strockbine and Galvin M. Kunin**

*Section of Infectious Diseases, Temple University Health Sciences Center, Philadelphia, Pennsylvania, U.S.A and** William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin, U.S.A.*

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Abstract □ Twenty six urea analogues, most of which have already been approved for human use, were tested for their antiurease activity *in vitro*. Cell-free extracts obtained from a clinical isolate of *Proteus mirabilis* was used as the source of enzyme. Acetohydroxamic acid which is a proven potent urease inhibitor but not approved for human use was again shown to be the most active compound among the tested. Phenacemide, cycloserine, and deferoxamine were demonstrated to be moderate inhibitors. Oxytetracycline, trimethoprim, and cefamandole revealed a demonstrable antirease activity, but only at very high concentrations.

The antiurease activity of cycloserine, trimethoprim, and cefamandole was pH dependent; only active at acidic pH. The inhibitory activity of acetohydroxamic acid however was independent of change in pH. Hydrogen ion concentration plays an important role in urease activity and acidification (pH 5.5) alone eliminates approximately 65% of the enzymic activity. Adjustment of pH therefore appears to be an important adjunct in reducing urease activity and should always be studied to maximize the efficacy of antiurease compounds under investigation.

Keywords □ Bacterial urease inhibitor, Antiurease compounds, *Proteus mirabilis*, pH effect on bacterial urease, Kinetics.

One of the most serious complications of urinary tract infections is formation of "infection stones", known as struvite and chemically as magnesium ammonium phosphate hexahydrate. This complication commonly occurs in the patient on long-term catheter drainage and in individuals with recurrent infections (9). Infection stones usually form when the conditions are such that the urine contains high concentrations of ammonia and the pH is alkaline. The microorganisms that produce urease, an enzyme catalyzing the hydrolysis of urea into ammonia and carbon dioxide, are mostly responsible for development of the urinary stones. Although Proteus species are best known for this property, a number of strains of Pseudomonas, Klebsiella, *Escherichia coli*, Staphylococcus, and Bacteroides also produce urease (9). An attractive approach to this problem has been the search of an effective urease inhibitor to prevent the formation of infection stones. Thus, numerous inorganic and organic compounds have been tested for their antiurease activities, but the findings have been rather disappointing (1, 2, 6). Of these compounds, acetohydroxamic acid has been one of the most potent and specific inhibitors of bacterial urease *in vitro* with promising results in animal studies (7). Nevertheless adequate data on human trial are lacking and continued search of a compound with antiurease activity that is effective and safe in human use is clearly indicated.

We have examined twenty-six different urea alalogues, most of which are already approved for human use, for their antiurease activities *in vitro*.

* Present address: Department of Pharmacy, Duk-Sung Women's College, Seoul 132, Korea

MATERIALS AND METHODS

Chemicals

Urea, tris (hydroxymethyl) aminomethane (Tham(R)), sodium hydroxide, hydrochloric acid, and maleic acid were obtained from Fisher Scien-

tific Company. Acetohydroxamic acid, hydroxyurea, phenytoin, pentobarbital, sulfaguanidine, oxytetracycline, thiouracil, thiourea, nitrofurantoin, 2-mercaptoethanesulfonic acid, and indigocarmine were purchased from Sigma Chemical Company, St. Louis, Mo. Other chemicals and antimicrobial agents, listed by their names, lot numbers where available, and suppliers were: Parachloromercuric benzoic acid (PCMB), Aldrich Chemical Co., Inc., Milwaukee, WI; phenacemide (Lot No. 856-7217), Abbott Laboratories, North Chicago, Ill.; trimethoprim (Lot No. 150), sulfamethoxazole (Lot No. 754017), Hoffman-La Roche Inc., Nutley, N.J.; metronidazole (Lot No. XB0750), Searle & Co., San Juan, Puerto Rico; oxacillin (Lot No. 77F600), Bristol Laboratories, Syracus, N.Y.; neomycin (Lot No. 582DK), penicillin G (3043Z), tolazamide, Lilly and Company, Indianapolis, ID; deferoxamine mesylate, Ciba Pharmaceutical Company, Summit, NJ; acetaminophen (Lot No. 7434), McNeil Laboratories, Inc., Fort Washington, PA; cefonicid sodium (Ipo No. 79253), Smith Kline French Labs., Philadelphia, PA; cefamandole nafate (Lot No. 4 DY95A), Lilly and Company, Indianapolis, ID; mercaptomerin sodium (Lot No. 4801974), Wyeth Laboratories, Inc., Philadelphia, PA.

Organism

A clinical isolate of *Proteus mirabilis* was grown at 37℃ in brain heart infusion broth (BBL, Becton, Dickinson & Co., Cockeysville, MD). Cells from 2-liter cultures were harvested by centrifugation at 4℃ and the supernate was discarded. The bacteria were washed twice with 0.1 M-Tris-maleate buffer, pH 7.0 and stored at −20℃ until use.

Preparation of Cell-Free Urease Extract

An ultrasonic lysis method was employed to obtain cell-free urease preparation. A 10 percent suspension of the organism in 0.1M-Tris-maleate buffer (pH 7.0) by wet weight was subjected to five one-minute ultrasonic treatments in a Sonifier Cell Disruptor (Model W-185C, Branson Sonic Power Co., Stamford, Conn.). The treatments were performed in an ice bath using standard tip, with power out put set at 60 watts. Cell debris were removed by centrifugation at 27,000×*g* for 20 minutes. The supernate was kept frozen in 5ml aliquots at −20℃ until use.

Urease Assay

The incubation mixture consisted of 0.5ml of the enzyme preparation, 0.4ml of 1M-urea and 0.1M-Tris-maleate buffer of pH under investigation to give a final volume of 10m*l*. Urease inhibitors under investigation were prepared in the buffer solution and added to the incubation mixture. The mixture was incubated at 37℃ for 1 hour, and the reaction was stopped by adding 0.1ml of 10M NaOH. The ammonia produced was measured by an Orion Analyzer Ammonia Electrode (model 95-10) as described by Byrne and Power (3). Ammonia standard solutions were made from a stock solution of 0.1M ammonium chloride (Orion Research Incorparated, Cambridge, Mass.) in a range of 10^{-1} to 10^{-5}M and similarly treated as the test mixture. Specific activity was expressed as millimoles of ammonia formed per gram of protein per hour. Protein was determined by the method of Lowry *et al* (11).

RESULTS

Effect of pH on Bacterial Urease

The enzyme activities were measured at various pH's ranging from 5.0 to 9.0 at 0.5 pH unit intervals. The results are represented in Figure 1. It is apparent that optimum pH for bacterial urease is pH 6.5-7.0 and the activity falls rapidly at either pH extremes.

Kinetics

The velocities of ammonia production during the incubation period (1 hour) were shown to be linear in relation to time. The Km of the enzyme for urea as determined by the usual double reciprocal plots (10) was shown to be 20-23 mM in Tris-maleate buffer (pH 7.0).

Measurements of Antiurease Activity

Twenty six different compounds, mostly urea or hydroxamic acid analogues, were tested for their antiurease activity. The assay system was the same as described above. Parachloromecuric benzoate and mercaptomerin sodium were employed as non-specific enzyme inhibitors and acetohydroxamic acid as a specific standard urease inhibitor. Their antiurease activities were screened at pH 7.0 and also tested at pH 5.5 and 8.5 when initial results revealed positive findings. The results are summarized in Table I.

Acetohydroxamic acid and hydroxyurea were the most effective inhibitors for the bacterial urease confirming previous findings (2, 6). Other compounds that gave demonstrable antiurease activity include phenacemide, tolbutamide, cycloserine, deferoxamine, and trimethoprim in order of decreasing potency on weight basis.

DISCUSSION

The primary objective of this study was to survey drugs that have already been approved for human

Table I. Antiurease Activity of Various Compounds.

Compounds	Concentrations mg/ml	Concentrations M	Per Cent Inhibition at pH 5.5	7.0	8.5
None (Control)		–	0	0	0
p-Chloromercuric benzoate	3.6×10^{-2}	1.0×10^{-4}	ND	100	ND
	3.6×10^{-3}	1.0×10^{-5}	ND	55	ND
Mercaptomerin sodium	6.0×10^{-1}	1.0×10^{-3}	ND	97	ND
Acetohydroxamic acid	7.5×10^{-2}	1.0×10^{-3}	91	96	93
	7.5×10^{-3}	1.0×10^{-4}	52	70	60
Hydroxyurea	3.8×10^{-1}	5.0×10^{-3}	95	95	97
	7.6×10^{-2}	1.0×10^{-3}	75	75	70
Phenacemide	6.0×10^{-1}	3.4×10^{-3}	ND	63	ND
	4.0×10^{-1}	2.2×10^{-3}	ND	33	ND
Cycloserine	2.0	2.0×10^{-2}	55	32	0
Trimethoprim	5.0	1.7×10^{-2}	75	31	0
Deferoxamine	4.0	7.0×10^{-3}	57	62	52
Cefamandole nafate	5.0	9.8×10^{-3}	26	27	0
Tolbutamide	2.0	7.4×10^{-3}	ND	65	ND
Tolazamide	2.0	6.4×10^{-3}	ND	30	ND
Oxytetracycline	3.0	6.5×10^{-3}	ND	50	ND
Neomycin	2.0	3.1×10^{-3}	9	27	10
Doxycycline	3.0	6.8×10^{-3}	ND	0	ND
Penicillin G	6.0	1.5×10^{-2}	ND	0	ND
Pentobarbital	2.0	8.0×10^{-3}	ND	0	ND
Phenytoin	1.0	4.0×10^{-3}	ND	0	ND
Nitrofurantoin	1.0	4.2×10^{-3}	ND	0	ND
Sulfaguanidine	2.0	1.0×10^{-2}	ND	0	ND
Sulfamethoxazole	2.0	4.0×10^{-3}	ND	0	ND
Cefonicid	5.0	1.2×10^{-2}	ND	0	ND
Mercaptoethanesulfonic acid	1.6	1.0×10^{-2}	ND	0	ND
Acetaminophen	3.0	2.0×10^{-2}	ND	0	ND
Thiourea	2.0	2.6×10^{-2}	ND	0	ND
Thiouracil	1.0	7.8×10^{-3}	ND	0	ND

use for their potential antiurease activity. Approximately 20 different compounds were tested using urease preparation obtained from a clinical isolate of *Proteus mirabilis*. The results are rather interesting in that there are a few points to be discussed. First of all, acetohydroxamic acid has been shown to be the most potent urease inhibitor among those tested. Other compounds that showed some activity at the concentrations tested include phenacemide, cycloserine (at acidic pH), deferoxamine, trimethoprim, and cefamandole nafate. Of these compounds, all but trimethoprim and cefamandole nafate contain either $-NH-\overset{\overset{O}{\|}}{C}-NH-$ or $-\overset{\overset{O}{\|}}{C}-NH-O-$ moiety as part of their chemical structure. It has previously been stated that $-\overset{\overset{O}{\|}}{C}-NH-OH$ is essential for a compound to exhibit a specific antiurease activity (8).

It should also be noted that acidic pH (i.e. 5.5)

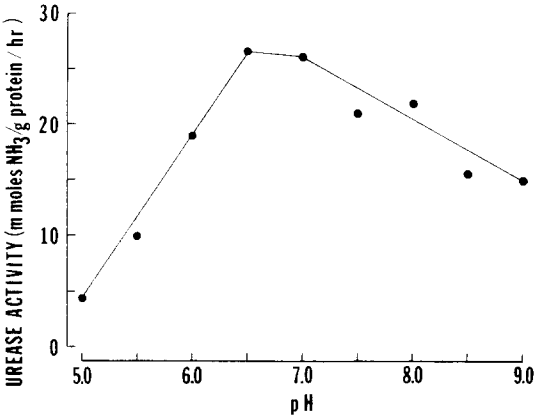

Figure 1. Effect of pH on Bacterial Urease. The assays were performed in 0.1M-Tris -maleate buffer with pH's as indicated. The enzymic activities are expressed as millimoles of ammonia formed per gram protein per hour. Other assay conditions and methods are described in detail in Materials and Methods.

is shown to be a very important adjunct in reducing urease activity. Although this is contrary to the findings of Rosenstein, *et. al.* (12), it is supported by other reports (8). One should be able to eliminate 60-70% of urease activity by simply adjusting the pH of urinary tract to 5.5 from 7.0-8.5 as demonstrated in Figure 1. Furthermore, it is obvious that the antiurease activity of cycloserine, trimethoprim, and cefamandole nafate is pH dependent and they lose their activity at alkaline pH. The inhibitory activity of acetohydroxamic acid however was independent of change in pH. It is obvious that acidification should be additive to the antiurease activity exerted by the inhibitor alone regardless of the pH dependence. Adjustment of pH therefore appears to be an important adjunct in reducing urease activity and should always be considred to maximize the efficacy of antiurease activity. It is noteworthy that the efficacy of lactulose in the treatment of chronic portalsystemic encephalopathy was highly associated with its ability to lower the stool pH to below 5.5 (4). This lactulose could exert triple effects in that the low pH should effectively inhibit the bacterial urease activity in the bowel, in addition to its abilities to trap the ammonia by the acidic metabolic products derived from it and to function as an osmotic diarrheal agent.

Although several compounds among the studied showed demonstrable antiurease activity, the concentrations necessary to achieve the activities are practically unobtainable under the normal clinical setting. Phenacemide has been shown to be one of better antiurease compounds tested, but it is mainly metabolized by the liver and no appreciable quantities of the drug are found in urine (5). Cycloserine and deferoxamine contain $-\overset{O}{\overset{\|}{C}}-NH-O-$ moiety in the structure, one and three per mole respectively, but the antiurease activities for both compounds were rather weak. Consequently continued search for effective antiurease agent with low toxicity is necessary.

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