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Inhibition of the Growth of *Ureaplasma urealyticum* by a New Urease Inhibitor, Flurofamide

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Flurofamide (N-[diaminophosphinyl]-4-fluorobenzamide), a urease inhibitor, was a potent inhibitor of the growth of *Ureaplasma urealyticum*. As little as 10 μM flurofamide (2 μg/ml) prevented any growth, but *U. urealyticum* survived for about eight hours before colony counts become undetectable. Flurofamide was a specific inhibitor of *U. urealyticum* since it did not inhibit growth of four *Mycoplasma* species or *Acholeplasma hippikon*. Flurofamide was 1,000 times more active than acetohydroxamic acid and thus has promise as a chemotherapeutic agent and a biochemical tool.

Urease is perhaps the most interesting protein currently, among the multitudinous proteins occurring in the organisms of the *Mycoplasmatales*. The enzyme and its substrate urea appear critical to *Ureaplasma urealyticum* because both the growth rate and the growth yield of the organisms in culture are functions of the urea concentration in the medium. The maximum growth rate is achieved at about 3 mM urea and the yield is directly related to the urea concentration up to 32 mM, provided that adequate buffering capacity is maintained [1]. Growth does not occur in the absence of urea. Compounds such as allantoin (and its derivatives) which appear to support growth [2], apparently do so because they spontaneously break down to urea [Kenny: unpublished data]. The apparent function of urease activity is to promote ATP synthesis by a possible chemiosmotic mechanism. Romano et al. [3] have shown that ATP synthesis is dependent upon the presence of urea and an active ATPase. ATP synthesis was blocked by the urease inhibitor, acetohydroxamic acid, indicating that urease was indispensable to ATP synthesis. The urea requirement of *U. urealyticum* is further remarkable in that no other organism is known to require urea in a rich medium: urea is required only by bacteria which possess urease, when it is the sole nitrogen source in defined media.

The enzyme appears to be a typical urease. The Km is about 5 mM urea and V_{max} is 26 mM urea with an optimum pH for activity in the 5.0-6.5 range [4,5]. The enzyme appears to be a major antigen of the species and the eight human serotypes studied all share the same serological specificity, which is distinct from that of jack-bean urease [Sayed, Kenny: in press]. The molecular weight of the active form obtained in SDS polyacrylamide gel electrophoresis is 140,000 daltons; upon boiling, an inactive band at 70,000 daltons appears [Sayed, Kenny: in press]. Several isoenzymes appear to exist [4,6].

"Flurofamide" is a new inhibitor of bacterial urease(s) designed to inhibit urease

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enzymes within intact cells [7]. In the present study, I report the inhibition of growth of *U. urealyticum* by small concentrations of flurofamide. This paper is a preliminary report of recent data, which I believe may have possible chemotherapeutic and biological significance.

MATERIALS AND METHODS

Inhibitors

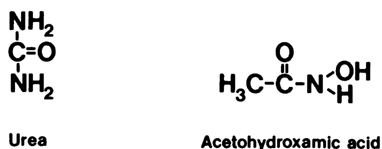
Both "flurofamide" (N-[diaminophosphinyl]-4-fluorobenzamide coded EU-4534, lot 6692-037A) and acetohydroxamic acid (coded EU-2350, lot 2883-A-90A) were gifts of Norwich-Eaton Pharmaceuticals (Norwich, NY). The structural formulae of flurofamide [7] and acetohydroxamic acid are shown in Fig. 1. Both compounds were soluble in water, and stock solutions were sterilized by filtration.

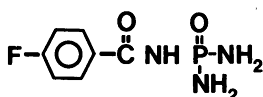
Organisms

U. urealyticum types 1,2,4, and 8 were obtained from J. Robertson; types 3 and 5 were ATCC (American Type Culture Collection) strains 27815 and 27817, respectively. Mycoplasma species were selected as representative of the seroclusters of mycoplasma species [8] and included: *Mycoplasma hominis* (ATCC 23114), *Mycoplasma gallisepticum* (ATCC 15302), *Mycoplasma* species bovine group 7 (N 29B), and *Mycoplasma felis* (B2). *Acholeplasma hippikon* (ATCC 29725) was used as a representative of genus *Acholeplasma. Proteus vulgaris* (ATCC 13315) was the only bacterial species tested.

Cultivation of Organisms and Sensitivity Testing

Growth curves of *U. urealyticum* were conducted as described previously [1]. Briefly, the broth medium employed was a dialysate of soy peptone and autoclaved fresh yeast. It was supplemented with 5 percent dialyzed horse serum, penicillin at 100 units per ml, 1 mM sodium sulfite, and was buffered with 20 mM 2-(N-morpholino)ethane sulfonic acid (MES buffer) at pH 6.3. Filter-sterilized urea was added at micromolar concentrations indicated in the text. Growth was assessed by determining colony-forming units (CFU) on MES buffered agar plates [1]. Antibiotic sensitivity of *U. urealyticum* was also determined by incorporating specific amounts of flurofamide or acetohydroxamic acid into MES agar, inoculating the plates with organisms, and observing for colonies (an agar dilution test procedure). A simple color-change method was used for screening concentrations. Medium con-





FLUROFAMIDE

N-[diaminophosphinyl]-4-fluorobenzamide

FIG. 1. Structural formulae of urea, acetohydroxamic acid, and flurofamide.

taining 5,000 μM urea, 20,000 μM MES buffer, and various concentrations of inhibitor was inoculated. An alkaline shift in pH as visualized by phenol red indicator was a reliable guide to significant growth of *U. urealyticum,* since the medium was heavily buffered. For determining flurofamide sensitivities of various mycoplasma species and *Acholeplasma hippikon,* agar plates were used which were similar in composition to MES agar plates but MES buffer was omitted and the pH was adjusted to 7.3.

RESULTS

Sensitivity of U. urealyticum *to Flurofamide*

Preliminary experiments showed that the growth of *U. urealyticum* type 8, as evidenced by ability to produce an alkaline reaction from phenol red in medium containing 5,000 μM urea was inhibited by 10 μM but not by 1 μM flurofamide. In order to determine whether flurofamide was bacteriocidal or bacteriostatic, growth curves were conducted using various concentrations of flurofamide and acetohydroxamic acid (Fig. 2). Flurofamide was a potent inhibitor of growth of *U. urealyticum:* no multiplication was observed at either 10 μM or 100 μM in the first eight hours, whereas control cells without flurofamide grew normally. At both 10 μM and 100 μM flurofamide, viability decreased to the limits of detection by 26 hours. At 1 μM flurofamide, the organisms grew well, though other experiments showed clear evidence of an increased generation time. A concentration of 10,000 μM acetohydroxamic acid was required to give suppression of growth similar to that obtained with 10 μM flurofamide. Even so, survival of the organisms was extended to 26 hours. At 1,000 μM acetohydroxamic acid, growth was slower than that of control cells without inhibitors. Color change was never observed in cultures which showed no evidence of growth by increase in CFU. Flurofamide appeared to be

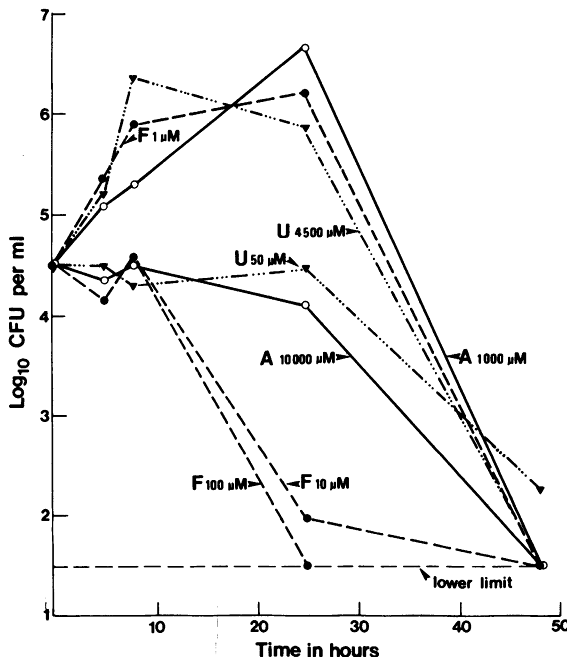


FIG. 2. Effect of flurofamide and acetohydroxamic acid on growth of *Ureaplasma urealyticum* type 8 in broth cultures. Medium: Soy peptone, fresh yeast dialysate broth supplemented with 20 mM MES buffer, 1 mM sodium sulfite, 5 percent dialyzed horse serum, and flurofamide and acetohydroxamic acid as indicated (in micromolar concentrations). Urea was included at 4,500 μM with all inhibitors and controls except in the one control where it was included at 50 μM (a concentration too low to give significant growth [1]). *Symbols:* **F** = flurofamide: **A** = acetohydroxamic acid: **U** = urea.

bacteriostatic (ureaplasmastatic), since no evidence of immediate killing was observed in this and other experiments. However, flurofamide was lethal to *U. urealyticum* in the sense that the inhibited organisms eventually became non-viable as evidenced by their inability to form colonies (Fig. 2). The initial inhibition of growth appeared little different from that obtained by lowering the urea concentration to 50 μM (Fig. 2), a concentration of urea which gives only survival [1].

Since the above experiment (Fig. 2) showed that the ability of flurofamide to prevent color change of the medium (inhibition of growth of *U. urealyticum*) was clear evidence of inhibition of the organism, a number of strains were tested for inhibition of growth in broth cultures. Types 1, 2, 3, 4, 5, and 8 were all inhibited by 10 μM but not by 1 μM flurofamide. When the same organisms were tested by agar dilution, colonies were visible microscopically on agar plates containing 1 μM but not 10 μM flurofamide. Therefore, determination of sensitivity of *U. urealyticum* to flurofamide appeared equivalent by the growth-curve, color-change, or agar dilution methods. A concentration of 10 μM flurofamide (~2 μg/ml) appeared sufficient to inhibit the growth of a number of strains of *U. urealyticum.*

Effect of Urea Concentration on Inhibition by Flurofamide

Since *U. urealyticum* can grow well in medium containing urea concentrations as high as 100,000 μM [1], various amounts of urea were added to media containing none, 1, 10, or 100 μM flurofamide. These were inoculated with *U. urealyticum* type 8 and the results assessed by both color change and CFU. Although 10 μM flurofamide consistently gave inhibition of growth in medium containing up to 10,000 μM urea, growth was not inhibited in media containing 100,000 μM. Both color change and growth were delayed but significant growth occurred, indicating that the inhibitory effect of flurofamide was affected by the urea concentration of the medium. When 100 μM flurofamide was employed, *U. urealyticum* did not grow even in the presence of 100,000 μM urea.

Effect of Flurofamide on Other Organisms

Proteus vulgaris grew well in the dialysate broth base without any supplementation and its growth was not inhibited by 100 μM flurofamide. The agar dilution method was used to assess inhibition of growth of mycoplasma species and *Acholeplasma hippikon;* none were susceptible to 10 μM flurofamide (see list in "Materials and Methods"). *Mycoplasma hominis* grew as rapidly in broth in the presence of 100 μM flurofamide as in its absence, as judged by increase in turbidity.

DISCUSSION

Flurofamide is a powerful inhibitor of growth of *U. urealyticum*: at 10 μM (~2 μg/ml), it is as inhibitory as many antibiotics [5]. It is 1,000-fold more active than acetohydroxamic acid (Fig. 2), a result which correlates exactly with the ratio between these two compounds as inhibitors of the urease activity of intact *Proteus* organisms [7]. My preliminary experiments also show that flurofamide is much more active as an inhibitor of jack-bean urease. Flurofamide appears to be bacteriostatic initially, but the organisms eventually die. Apparently, it functions by depriving the organisms of an essential nutrient, urea. The fact that it is active in very small quantities and specific to *U. urealyticum* argues that the hydrolysis of urea is critical to growth of the organism. Since the inhibition produced by 10 μM flurofamide can be alleviated by increasing the urea concentration to 100,000 μM (a 10,000:1 ratio), the inhibition appears specific to urea and likely to urease activity.

The molecule has a structure similar to the urea molecule (right side of molecule, Fig. 1) which probably binds in the combining site of urease. If the fluorine binds to some amino acid near but not in the combining site, this might stabilize the bond and explain the great activity of flurofamide. If urease activity is essential to *U. urealyticum,* then it may not be possible to obtain a resistant mutant to flurofamide. However, if a urease-less mutant of *U. urealyticum* is biologically possible, flurofamide would prove an excellent tool for its selection. Either result would be of great interest.

Flurofamide not only has promise as a biochemical tool but it may also be clinically useful as well. It appears reasonably clear that *U. urealyticum* has a role in human and animal disease, albeit as an opportunist [9,10]. The fact that flurofamide is as active as an antibiotic and likely ruthlessly specific to *U. urealyticum* suggests that it might be a useful chemotherapeutic agent. Flurofamide has been developed as a potential agent for the prevention of formation of kidney stones [7]. Apparently, human trials have not been attempted yet, but they may be in the future. Flurofamide has an LD_{50} for mice of 2.125 g/kg and possible human dosages of less than 5 mg/kg have been suggested for prevention of kidney stones [7]. It is of interest to note that acetohydroxamic acid has been tried in humans for that purpose [11]. If flurofamide or some similar compound were to be useful in humans, the probable great specificity of flurofamide would be most useful in specifically treating ureaplasmic infections and in determining which infections are actually caused by *U. urealyticum.* The partial reversal of the inhibitory effect of 10 μM flurofamide by 100,000 μM urea is of clinical interest since mammalian blood levels range from 20,000 to 40,000 μM urea and the concentration of urea in urine reflects the amount of urea in the blood. Clearly, some *in vivo* model is needed for further evaluation of flurofamide and similar compounds. If urease or its products are toxic [12], then flurofamide not only might eliminate the organism but also alleviate immediate toxic reactions. Finally, flurofamide may be important in clinical microbiology as a selective agent for the elimination of ureaplasmata much as lincomycin eliminates large colony forms [5].

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