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Bioevaluation of the Antibacterial Flumequine for Urinary Tract Use

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The antimicrobial activity of flumequine (R-802) was characterized by in vitro and in vivo procedures. Assay of the minimal inhibitory concentrations for 321 recent clinical isolates revealed that 88% of the gram-negative bacteria were inhibited by an R-802 concentration of 6.2 μg/ml or less. Cross-resistance in laboratory-derived mutants of *Proteus vulgaris* was essentially complete for R-802, nalidixic acid, and oxolinic acid, although quantitative differences were evident. R-802 was more effective than either of these quinolone antibacterials in preventing the development of experimental murine pyelonephritis (*P. vulgaris*). R-802 and trimethoprim/sulfamethoxazole (1:5) were equally effective in resolving a *P. mirabilis*-induced prostatitis of rats.

Flumequine (R-802) is a synthetic antibacterial, selected from a series of tricyclic quinolines prepared in Riker Laboratories. The compound is 6,7-dihydro-9-fluoro-5-methyl-1-oxo-1H,5H (ij) quinolizine-2-carboxylic acid (Fig. 1). This report describes the bioevaluation of R-802 and its comparison with several antimicrobials in clinical use.

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MATERIALS AND METHODS

Bacteria. Laboratory stock cultures were used. Susceptibility testing of clinical isolates was accomplished with cultures from urine specimens taken at the St. Paul Veterans Administration Hospital, St. Paul, Minn. These isolates were identified using conventional microbiological methods (1). Wound isolates of *Staphylococcus aureus* were obtained from the St. Louis Park Clinic, Minneapolis.

Antimicrobials. Flumequine (R-802) was synthesized in Riker Research Laboratories. Stock solutions of R-802 (1%) for in vitro use were prepared with 0.1 N sodium hydroxide. Nalidixic acid and oxolinic acid were provided by Winthrop Laboratories and Warner-Lambert Research Institute, respectively. Bactrim (sulfamethoxazole and trimethoprim, 5:1), Erythrocin (erythromycin stearate), and Terramycin (oxytetracyline-hydrochloride) were obtained in commercial form.

Susceptibility. The antimicrobial spectrum of R-802 was surveyed on tryptone soy agar (TSA) plates with a $\log_{10}$ dilution series. Microliter aliquots of stock solution were mixed with molten TSA in individual screw-cap vials and dispensed into petri dishes. These preparations were allowed to harden and were surface inoculated with stock culture suspensions. The conditions of incubation were overnight at 37 C in a humidified atmosphere enriched with 10% carbon dioxide. Growth inhibition of those strains that appeared to be most susceptible to R-802 was further examined by a $\log_2$ dilution tube technique in nutrient broth (5). Nalidixic acid and oxolinic acid were included in this titration as reference quinolone antibacterials. *Bacillus subtilis* was deleted in this comparison because of pellicle formation in static broth culture; *Proteus vulgaris*, which was not tested on TSA because of the "swarming" phenomenon, was included here. The minimal inhibitory concentrations (MICs) of R-802 for clinical isolates of urinary tract infection were similarly determined; the influence of culture medium pH was examined in selected strains at 0.2-unit intervals over the range 6.0 to 8.0.

Cross-resistance of mutant clones selected by growth on oxolinic acid, nalidixic acid, and R-802 was assessed by the replica plating technique (6). Approximately 10^9 cells were spread over the surface of drug plates containing TSA plus 0.15% bile salts to inhibit swarming of *P. vulgaris* 210. The levels of drug permitting development of approximately 10 colonies/plate were as follows: R-802 and oxolinic acid, 10 μg/ml; nalidixic acid, 50 μg/ml. No colonies were observed on plates containing 25 μg of R-802 or oxolinic acid per ml. An imprint of the colonies developing on each original plate was transferred to sterile velveteen templates. These in turn were used to reproduce the patterns on several test plates containing the individual drugs. All plates were scored after 2 days of incubation at 37 C.

Efficacy. Preclinical evaluation of R-802 for treatment of urinary tract infection used a rat model, which mimics the development of bacterial pyelonephritis in humans (8). One-tenth milliliter of an overnight shake culture of *P. vulgaris* 210 was injected into the partially ligated bladder of female Simonsen rats. Tryptone soy broth was used as the growth medium and diluent. The temperature of

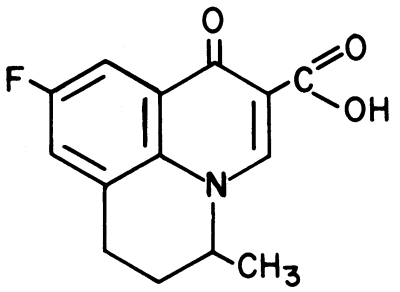

FIG. 1. *Structure of flumequine (R-802).*

incubation was 35 to 37 C with rotary agitation at 200 rpm. The highest dilution (usually 1:10) producing uniform bladder infections, which persisted for at least 10 days, was used. Antimicrobials were suspended in 4% aqueous acacia and homogenized with a ground-glass tissue mill. Treated animals were dosed daily by oral gavage beginning 24 h postinfection for a period of 3 weeks. Treatment periods of only 10 days at elevated doses (namely, 100 mg of R-802/kg per day) were inadequate based on culture of excised tissue. Infection control animals received no treatment. Water was supplied ad libitum. Animals were sacrificed 24 h post-treatment to culture both kidneys and the urinary bladder. Kidney slices and bladder fragments were placed on TSA agar to reveal the characteristic swarming outgrowth in positive cultures. No antibacterial activity could be demonstrated in excised tissues by using sensitive indicator bacteria. Infection relapse was assessed by retaining animals for a period of 2 weeks post-treatment prior to sacrifice and culture.

An experimental prostatitis was induced in male Simonsen rats according to the method of Friedlander and Braude (4). The growth conditions for *P. mirabilis* 120 were the same as those described for *P. vulgaris* 210. The undiluted culture in a nonrefluxing volume (0.05 ml) was injected into the unrestricted bladder of surgically prepared animals. Antimicrobials were suspended in 4% aqueous acacia and homogenized with a ground-glass tissue mill. Treated animals were dosed daily by oral gavage beginning 5 days postinfection for a period of 3 weeks. Infection control animals received no treatment. Water was supplied ad libitum. Animals were sacrificed after a total of 25 days to culture the ventral prostate, the urinary bladder, and both kidneys on TSA plates; the dorsolateral prostate is less frequently involved and was not monitored for evidence of infection.

Urinary levels of active drug were estimated by agar diffusion assay using *Klebsiella pneumoniae* 113 as the indicator bacterium. In lieu of cylinders, a stainless-steel template containing six wells (bioassay plate no. 1310, Labline Instruments, Inc.) was placed on the surface of seeded TSA plates. R-802 standards and pooled urine samples were diluted in tryptone soy broth. Each rat (three per group) received a single dose of 10 or 25 mg of R-802 per kg prior to 24-h urine collection.

RESULTS

In vitro. The susceptibility to R-802 of selected strains of microorganisms is presented in Table 1. The concentrations of R-802 inhibiting growth of bacteria ranged from 1 to 100 μg/ml, whereas no antifungal activity was evident at 100 μg of R-802 per ml. Antibacterial activity was unchanged by the addition of 10% (vol/vol) horse serum to the TSA growth medium.

The MICs of quinolone antibacterials presented in Table 2 suggested that a study of the response of recent clinical isolates from urinary tract infection to R-802 should be pursued. No "skip-tube" end points indicating rapid selection of resistant progeny were evident in this experiment.

Assay of the R-802 MICs for 321 recent clinical isolates (Table 3) revealed that 88% of the gram-negative bacteria were inhibited at a concentration of 6.2 μg/ml or less. The majority of gram-positive strains exhibited an MIC of 12.5 μg/ml or more. A different response to R-802 by these two groups of bacteria was also suggested by the influence of growth medium pH on antimicrobial activity in broth culture. The MIC values for *Escherichia coli* VA-1, *P. vulgaris*

TABLE 1. *Antimicrobial activity of R-802*[a]

Microorganism	MIC (μg/ml)	
	Serum free	10% Horse serum
Streptococcus pyogenes 114	100	100
Staphylococcus aureus 1	10	10
Bacillus subtilis 6	1	1
Escherichia coli 22	1	1
Pseudomonas aeruginosa 12	100	100
Streptococcus faecalis 196	100	100
Klebsiella pneumoniae 197	10	10
Aspergillus niger 18	>100	>100
Candida albicans 20	>100	>100

[a] Growth inhibition of laboratory stock cultures determined by TSA plate dilution.

TABLE 2. *MICs of quinolone antibacterials*[a]

Bacterium	MIC (μg/ml)		
	Nalidixic acid	Oxolinic acid	R-802
Proteus vulgaris 210	12.5	0.8	0.8
Klebsiella pneumoniae 197	0.8	0.4	0.4
Escherichia coli 22	3.1	0.4	0.8
Staphylococcus aureus 1	12.5	1.6	1.6

[a] Growth inhibition of laboratory stock cultures determined by the serial dilution tube technique in nutrient broth.

TABLE 3. *Cumulative percentage of clinical isolates inhibited at various R-802 concentrations*[a]

Bacterium	MIC (μg/ml):							Total isolates
	0.8	1.6	3.1	6.2	12.5	25.0	>25	
Escherichia coli	26	48	84	100	–[b]	–	–	58
Proteus vulgaris	–	14	43	71	100	–	–	7
Proteus mirabilis	8	28	58	80	100	–	–	40
Proteus rettgeri	14	29	43	71	100	–	–	7
Proteus morganii	35	57	78	87	100	–	–	23
Klebsiella pneumoniae	2	9	39	78	100	–	–	64
Enterobacter aerogenes	–	7	46	96	100	–	–	28
Pseudomonas aeruginosa	–	4	17	79	88	100	–	24
Serratia marcescens	12	44	75	100	–	–	–	32
Providence	17	50	50	83	100	–	–	6
Streptococcus faecalis	–	–	–	4	62	85	100	26
Staphylococcus aureus[c]	–	–	–	–	100	–	–	6

[a] Growth inhibition determined by the serial dilution tube technique in nutrient broth.
[b] –, Zero incidence.
[c] Wound isolates.

VA-1, and *P. mirabilis* VA-3 were invariant in the pH range 6.0 to 8.0; the MIC for *S. aureus* 148 increased 10-fold to a maximum of 25.0 μg/ml at pH 8.0.

Cross-resistance in laboratory-derived mutants of *P. vulgaris* was essentially complete for R-802, nalidixic acid, and oxolinic acid (Table 4). Although discrete colonies were evident on nalidixic acid selection plates containing 10 μg of drug per ml, a background lawn of additional mutants was confirmed on replica plates. Multistep mutants selected by serial transfers in increasing concentrations of each drug confirmed these observations: cross-resistance was demonstrated for *P. vulgaris* clones capable of growing on TSA containing 100 μg of R-802, 100 μg of oxolinic acid, or 500 μg of nalidixic acid per ml.

In vivo. the R-802 dose response obtained in an ascending rat urinary tract infection model is presented in Table 5. The median effective dose based on several titrations in this model was approximately 10 mg/kg per day. The data were not cumulatively scored as the sum of all positive cultures for each dose level, because upon cessation of treatment animals with infected bladders would surely develop infected kidneys in due course. A variable number of animals succumbed to fatal infections prior to terminating the experiment, as indicated in the column of survivors.

The comparative efficacy of R-802 and two urinary tract antimicrobials is presented in Table 6. The performance of nalidixic acid was comparable to that of oxolinic acid, whereas R-802 was clearly more effective in preventing the development of pyelonephritis as defined in this model. Bladder isolates from the two R-802 treatment failures were indistinguishable from the infecting culture in terms of antimicrobial susceptibility.

The absence of infection relapse in a short-term follow-up experiment is evident in Table 7. Bladder isolates from the four rats treated

TABLE 4. *Cross-resistance of P. vulgaris clones isolated in the presence of certain quinolone antibacterials*

Original selection plate			Cross-resistant clones on test replica plate			
Quinolone	Concn (μg/ml)	No. of colonies	Oxolinic acid (10 μg/ml)	Nalidixic acid		R-802 (10 μg/ml)
				10 μg/ml	50 μg/ml	
Oxolinic acid	10	12	16	16	16	13
	10	11	12	12	12	11
Nalidixic acid	10	~44	10	TNTC[a]	TNTC	4
	50	9	4	TNTC	9	4
R-802	10	13	12	12	12	9
	10	7	7	7	7	2
	10	9	5	6	5	6

[a] TNTC, Too numerous to count.

TABLE 5. *Ascending rat urinary tract infection model: titration of R-802*[a]

Group	Survivors (21 days)	Left kidney	Right kidney	Bladder
Infection controls (*P. vulgaris*)	8/10	6/8	6/8	7/8
Infected and treated with R-802 (mg/kg per day)				
100	10/10	0/10	0/10	1/10
75	10/10	0/10	0/10	0/10
50	10/10	0/10	0/10	0/10
25	10/10	0/10	0/10	0/10
10	9/10	2/9	1/9	4/9

[a] Each group consisted of 10 animals; the last three columns indicate the ratio of positive cultures to survivors.

TABLE 6. *Ascending rat urinary tract infection model: comparison of R-802 with reference quinolones*[a]

Group	Survivors (21 days)	Left kidney	Right kidney	Bladder
Infection controls (*P. vulgaris*)	8/10	7/8	7/8	7/8
Infected and treated with:				
R-802, 25 mg/kg per day	10/10	0/10	0/10	2/10
Nalidixic acid, 25 mg/kg per day	9/10	2/9	4/9	4/9
Oxolinic acid, 25 mg/kg per day	9/10	3/9	4/9	6/9

[a] Each group consisted of 10 animals; the last three columns indicate the ratio of positive cultures to survivors.

with 25 mg of R-802/kg per day were again unchanged in terms of R-802 susceptibility.

The therapeutic response to R-802 and three reference antimicrobials in a *P. mirabilis*-induced prostatitis is presented in Table 8. In three experiments, the incidence of positive prostate cultures from infection control groups of 10 animals each ranged from 0.72 to 0.86; positive cultures from both kidneys of each animal closely paralleled the incidence of prostate infection. The incidence of bladder infection was slightly higher, as indicated. R-802 was essentially ineffective in this model at a dose of 25 mg/kg per day. A dose of 50 mg/kg per day resulted in a 65% reduction in the incidence of prostatitis and pyelonephritis, as measured by in vitro culture of excised tissue. Oral administration of erythromycin and trimethoprim/sulfamethoxazole significantly reduced the incidence of positive prostate cultures at 50 and 100 mg/kg per day. These reductions were not closely paralleled by results obtained from tissues of the urinary tract per se. Oxytetracycline was poorly effective in this model by comparison with the other antimicrobials examined.

Based on the in vitro activity of R-802, the levels of active drug achieved in a 24-h urine collection from rats receiving a single oral dose were 15 μg/ml at 10 mg of R-802 per kg of body weight and 41 μg/ml at 25 mg of R-802 per kg of body weight.

TABLE 7. *Ascending rat urinary tract infection model: infection incidence 14 days post-treatment*[a]

Group	Time (days)	Survivors	Left kidney	Right kidney	Bladder
Infection controls (*P. vulgaris*)	21	5/10	4/5	4/5	4/5
Infected and treated with R-802 (mg/kg per day)					
50	21	7/7	0/7	0/7	0/7
50	21 (+14)	7/8	0/7	0/7	0/7
25	21	6/8	2/6	1/6	3/6
25	21 (+14)	6/7	1/6	0/6	1/6

[a] The infection control group consisted of 10 animals; 15 animals were treated in each dosage group as indicated. The last three columns indicate the ratio of positive cultures to survivors.

DISCUSSION

Quinolone antimicrobials are broad-spectrum agents reserved primarily for urinary tract use. Therapeutic advantages for *Proteus* sp. infections, in particular, were suggested in a comparative study of 2,081 clinical isolates which used Neg-gram sensitivity disks (9). In one study of urinary tract infections, nalidixic acid performed significantly better than sulfadimidine, producing cure rates of 93.7 and 81.4%, respectively; this result led the authors to regard nalidixic acid as a drug of choice when bacteriological facilities are not available (2). It is also clear that development of resistant organisms after administration of existing quinolones is a frequent problem (3,7). There is an

TABLE 8. *Efficacy of R-802 and reference antimicrobials in P. mirabilis-induced prostatitis*[a]

Treatment	Survivors	Prostate	Bladder	Right kidney	Left kidney
Infection controls	24/30	19/24 (0.79)	22/24 (0.92)	20/24 (0.83)	20/24 (0.83)
Infected and treated with R-802 (mg/kg per day)					
25	10/10	7/10 (0.70)	8/10 (0.80)	8/10 (0.80)	8/10 (0.80)
50	29/30	8/29 (0.28)	16/29 (0.55)	9/29 (0.31)	9/29 (0.31)
100	10/10	0/10	0/10	0/10	0/10
Erythromycin (mg/kg per day)					
50	7/10	1/7 (0.14)	6/7 (0.86)	6/7 (0.86)	6/7 (0.86)
100	8/10	2/8 (0.25)	6/8 (0.75)	4/8 (0.50)	2/8 (0.25)
Oxytetracycline (mg/kg per day)					
50	10/10	5/10 (0.50)	6/10 (0.60)	6/10 (0.60)	6/10 (0.60)
100	9/10	4/9 (0.44)	7/9 (0.78)	6/9 (0.67)	6/9 (0.67)
Trimethoprim/sulfamethoxazole (mg/kg per day)					
50	8/10	3/8 (0.38)	8/8 (1.00)	7/8 (0.88)	6/8 (0.75)
100	10/10	1/10 (0.10)	6/10 (0.60)	2/10 (0.20)	4/10 (0.40)

[a] Survival ratios indicate the number of animals that succumbed to infection between day 5, when treatment was initiated, and day 25, when specimens were taken. The last four columns indicate the ratio of positive cultures to survivors; the incidence of infection calculated from this ratio is shown in parentheses.

apparent absence of transferable drug resistance with these compounds, due in part to the prescribed indications. Laboratory-induced mutations to quinolone resistance occur in stepwise fashion, and the synthesis of oxolinic acid that enhanced in vitro activity improved the potential therapeutic value of these compounds. The results obtained in the present study utilizing appropriate rodent models demonstrate the added potential of R-802.

In vitro testing of R-802 demonstrated significant antibacterial activity for the principal pathogens in urinary tract infection. In addition to *E. coli*, *Proteus* sp., and the *Klebsiella-Enterobacter* group, the clinical isolates examined in this study also included 32 *Serratia marcescens*; *S. marcescens* is usually regarded as an index of nosocomial infection and appeared to resemble *E. coli* in terms of R-802 susceptibility. The pronounced influence of pH on antibacterial activity versus *S. aureus* has been observed with other quinolones and is unexplained at present.

In vivo testing of R-802 was aimed at determining efficacy for intended use. The *Proteus* sp.-induced pyelonephritis is a good model for comparing quinolone antibacterials for reasons previously cited. It is less useful for antibiotics, e.g., ampicillin, but attempts to substitute other infecting bacteria have met with limited success. Prostatitis can be a significant treatment problem and source of chronic bacteruria (10). The rodent model used to challenge R-802 for this indication is idealized in terms of the infecting agent; nevertheless, the method provides a measure of antimicrobial efficacy rather than antimicrobial activity partitioned into prostatic secretions. The reference antimicrobials in this experiment were selected largely on the basis of clinical interest. Oxytetracycline hydrochloride would be a poor choice based on in vitro susceptibility of most strains of *P. mirabilis* (9). The in vitro activity of erythromycin stearate is favored by slightly alkaline conditions, and the pH of mammalian prostatic fluid is usually less than 7.0. Nevertheless, erythromycin was effective in eliminating bacteria from the prostate. The therapeutic responses to R-802 and trimethoprim/sulfamethoxazole were dose related and comparable for prostatitis. Additional indications for R-802 have been investigated in this laboratory and will be reported in a separate communication.

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