Aryloxoalcanoic compounds induce resistance to antibiotic therapy in urinary tract infection caused by *Escherichia coli*

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Abstract

Clofibric acid (CL) is a compound used to control hypertriglyceridemia, and ethacrynic acid (ET) is administered to enhance diuresis. These compounds are structurally analogous to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), as they have a chlorinated phenoxy moiety. As these agents are mainly excreted by the renal route, they could potentially coexist with *Escherichia coli* in the urinary tract of infected patients. Induction of the *in vitro* resistance of *E. coli* to hydrophilic antibiotics was determined by increasing the values of the minimum inhibitory concentration (2–40-fold). These results correlated with drastically inhibited expression of the hydrophilic bacterial channel OmpF. *In vivo* assays were performed in ascending urinary tract infection in female BALB/c mice. Treatment with the hydrophilic antibiotic cephalexin $25\,mg\,kg^{-1}\,day^{-1}$ by the oral route diminished renal infection. The CFU mean values in the kidneys were between 75% and 89% lower than those in animals without treatment. Simultaneous exposure to CL (at a therapeutic dose, $28.6\,mg\,kg^{-1}\,day^{-1}$) did not change the effect of the treatment. In contrast, ET at $2.9\,mg\,kg^{-1}\,day^{-1}$ or 2,4-D at $70\,mg\,kg^{-1}\,day^{-1}$ inhibited the antibiotic therapeutic effect. Moreover, 2,4-D dramatically increased bacterial infection after 9 days of exposure.

Introduction

Bacteria utilize several mechanisms to develop resistance to antibiotics. Some are specific for a single drug or class of drugs (inactivation of the drug by enzymatic modification and alteration of the drug target), and others are more general mechanisms in which access of the agent to the target is prevented by the barrier and active transport functions of membranes (Nikaido, 1994; Spratt, 1994; Ochs *et al.*, 1999). Whereas some of them account for bacterial intrinsic resistance, the expression of others is regulated in response to environmental changes in temperature, oxidation–reduction state or the presence of drugs (Aronoff, 1988; Rosner *et al.*, 1991; Chou *et al.*, 1993; Cohen *et al.*, 1993; Nikaido, 1996). Previous reports showed that salicylate and other membrane-permeating weak acids, such as acetate and benzoate, added to culture medium in susceptibility tests showed opposite effects, depending on the antibiotic tested. Whereas salicylate causes potentiation of susceptibility to aminoglycosides in *Escherichia coli*, the same drug increases resistance to several antibiotics, such as ampicillin, tetracycline, chloramphenicol, nalidixic acid and cephalosporins (Rosner, 1985; Foulds *et al.*, 1989; Aumercier *et al.*, 1990).

Aryloxoalcanoic acids (AOAs) comprise a family of aromatic weak acids that includes: clofibric acid (CL), the prototypical hypolipidemic fibrate from a group of pharmaceutical products administered in the treatment of hypertriglyceridemia (Witztum, 1996); ethacrynic acid (ET), which has a diuretic action at the level of the ascending limb of Henle (Jackson, 1996); and the widely used selective herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Taskar *et al.*, 1982; Knopp & Glass, 1991). These compounds are mainly excreted unaltered or conjugated by the renal route (Taskar *et al.*, 1982; Knopp & Glass, 1991; Jackson, 1996; Witztum, 1996). We previously described the effects of 2,4-D on urinary tract infection (UTI), using an exposure protocol in which two doses were administered prior to infection in a murine experimental model (Balagué *et al.*, 2002). In addition, we described the bacterial cell surface alterations caused by 2,4-D *in vitro*, specifically a decrease of fimbrial and total protein contents (Balagué *et al.*, 2001).

The lack of information on *in vitro* and *in vivo* antibiotic activity in the presence of aryloxoalcanoic compounds and their possible coexistence with gram-negative bacteria in urine during UTI led us to investigate the influence of 2,4-D, CL or ET on therapy for *E. coli*. In the present work, we studied: (1) the *in vitro* susceptibility to antibiotics of *E. coli* in the presence of AOAs; and (2) the *in vivo* pattern of UTI treatment during exposure to these compounds, using a murine model of ascending infection.

Materials and methods

Strains

Escherichia coli ATCC 35218 and ATCC 25922 are standard strains for antibiotic susceptibility tests. *Escherichia coli* RM11, RM3518, RM2608, T149 and RM6 are clinical isolates from patients with UTI, selected from a collection of 100 uropathogenic *E. coli* strains obtained in the Microbiological Departments of the National Universities of Rosario and Tucumán, Argentina. Clinical isolates were identified to the species level by conventional bacteriologic methods (Orskov, 1986). The nonpathogenic *E. coli* strain was HB101. Outer membrane proteins (Omps) extracted from mutant strains AW738 (OmpF+ OmpC−) and AW739 (OmpF− OmpC+) and the isogenic *E. coli* strain AW737 (Ingham *et al.*, 1990) were used as markers in Omp profiles. Isolates were cultured on blood agar, incubated aerobically at 37 °C for 24 h, and transferred to 20% glycerol broth for storage at −70 °C. Strains were recovered by subculture on blood agar at 37 °C.

Chemicals and growth media

Mueller–Hinton broth (MHB), Cysteine Lactose Electrolyte Deficient (CLED) agar, McConkey agar and antibiotic disks were obtained from Difco Laboratories (Detroit, MI), chloramphenicol was obtained from Calbiochem, Novabiochem Corporation (La Jolla, CA), norfloxacin was obtained from Laboratorios Bagó (Bs As, Argentina), cephalexin was obtained from Argentia, Bristol-Myers Squibb (Bs As, Argentina), and penicillin G, cefotaxime, cephalothin, trimethoprim, tetracycline, rifampicin, sodium *N*-lauroyl sarcosinate (sarkosyl), ET, CL, 2,4-D, 2,4-D sodium salt, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents and all other chemicals and solvents were purchased from Sigma Chemicals Co. (St Louis, MO) and Merck (Bs As, Argentina).

Susceptibility testing

Susceptibility to amoxicillin, amoxicillin–clavulanic acid, ticarcillin, ticarcillin–clavulanic acid, cephalothin and cefoxitin was tested by the Kirby–Bauer disk diffusion method (Bauer *et al.*, 1966) to determine β-lactam resistance phenotypes (Henquell *et al.*, 1994).

β-Lactamase assays

β-Lactamase production was screened in clinical isolates with 6000 μg mL^{-1} of penicillin G as the substrate in phosphate buffer (pH 6). After 30 min of incubation, the assay results were revealed with 1% w/v soluble starch and a 2.03%/53.2% w/v solution of iodine/iodure (Swenson *et al.*, 1995).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations

The standard broth dilution method, as specified by the National Committee for Clinical Laboratory Standards (1997), in MHB without cation supplementation was used for MIC determination with a final inoculum of 10^5 CFU mL^{-1} of exponentially growing cells. The MIC was determined after 18 h of incubation at 37 °C. The antibiotics assayed are representative of unrelated classes of drugs: trimethoprim, norfloxacin, chloramphenicol, tetracycline, rifampicin, cephalothin and cefotaxime. The MIC was recorded as the lowest concentration of antibiotic that had completely inhibited visible growth of the bacteria. MBCs were determined by plating broths showing no turbidity onto control blood agar plates. The MBC was recorded as the lowest concentration of antibiotic with a lethal effect on over 99.9% of the initial inoculum.

In similar conditions, the influence of AOAs on MICs and MBCs was determined by adding 2,4-D, CL or ET at three different concentrations (1, 0.1 or 0.01 mM) to the MHB.

Pre-exposure experiments

An inoculum of 10^8 CFU mL^{-1} of the selected strains was grown in the presence of 2,4-D (1 or 0.01 mM) in successive pre-exposures during five complete growth cycles (18 h) in MHB, and washed in 0.9% NaCl before inoculation for MIC and MBC determinations, performed as described above.

Preparation of outer membranes

Omps were prepared by the sarkosyl solubilization method of (Lambert, 1988). Strains were grown aerobically for 24 h in MHB, harvested by centrifugation, and washed twice in phosphate-buffered saline (8 g L^{-1} NaCl; 0.2 g L^{-1} KCl; 0.2 g L^{-1} KH$_2$PO$_4$; 2.9 g L^{-1} Na$_2$HPO$_4 \cdot 2$H$_2$O; pH 7.4) at 4 °C. Bacterial pellets were suspended in 5 mL of distilled water, and cells were disrupted by 10×30 s pulses of sonication in an ice bath, with 30 s intervals for cooling. Unbroken cells were removed by centrifugation at 5000 *g* for 5 min and discarded. The supernatant was mixed with

0.5 mL of 22% w/v sodium *N*-lauroyl sarcosinate. After incubation for 30 min at room temperature, the mixture was centrifuged at 100 000 *g* for 45 min, and the pellet was washed twice in distilled water and stored at − 70 °C. The protein content of samples was determined by the method of Bradford (1976), using bovine albumin as standard.

The same procedure was used for bacteria grown in MHB supplemented with 2,4-D, CL or ET (1 mM).

SDS-PAGE

This was performed on 12% (w/v) acrylamide gels, supplemented with 8 M urea, using the (Laemmli, 1970) system. Samples were mixed with an equal volume of denaturing buffer (50 mM Tris-HCl, 2% w/v SDS, 10% v/v glycerol and 1% v/v β-mercaptoethanol) and boiled for 2 min at 100 °C prior to electrophoresis. Gels were stained with Coomasie Brilliant Blue R-250 in methanol/water/acetic acid (50 : 40 : 10) and destained in water/methanol/acetic acid (83 : 10 : 7).

In vivo assays

The Ethical Committee for Animal Care of the Reference Center for Lactobacilli (CERELA-CONICET) approved the experimental protocol used. Two-month-old female BALB/c mice from the breeding colony of the Microbiological Department of the National University of Tucumán were used throughout the investigation. The animals were housed in plastic cages and fed *ad libitum*, with their environmental conditions being kept constant. Each experiment was carried out with a group of 25–35 mice. Urine was cultured 24 h prior to challenge, and mice containing bacteria at levels higher than 10^2 CFU mL^{-1} were excluded. Uropathogenic *E. coli* strain T149 was inoculated intraurethrally in a 0.5% peptone water suspension (0.05 mL) at an infectious concentration (10^7–10^8 CFU mL^{-1}). A plastic polyethylene catheter (0.5 mm in diameter) coupled to a syringe was used for this purpose.

Therapeutic assay

The suspension of *E. coli* was inoculated as a single application 1 day before the first dose of each AOA. 2,4-D sodium salt, ET or CL was administered orally every day (50 μL). Cephalexin treatment started 10 h after the first AOA dose, with a dose of 25 mg kg^{-1} day^{-1} by the oral route. The studies were performed in the following groups of mice: group I, control mice challenged with *E. coli*; group II, control of treatment mice inoculated with bacteria and treated with cephalexin; group III, reference groups treated with 2,4-D at 70 mg kg^{-1} day^{-1}, CL at 28.6 mg kg^{-1} day^{-1} (therapeutic dose), or ET at 2.9 mg kg^{-1} day^{-1} (therapeutic dose); and group IV, the study groups challenged with

E. coli, exposed to AOAs at the different doses described, and given antibiotic treatment.

The 2,4-D concentrations used *in vitro* and *in vivo* were chosen on the basis of our previous studies (Balagué *et al.*, 2002, 2001).

Bacterial counts in tissue homogenate

The animals were killed by cervical dislocation. Kidneys were removed aseptically, placed in 0.5% peptone/water, and homogenized with a Teflon pestle. Dilutions were made, and the samples were plated onto MacConkey agar plates. The plates were incubated for 48 h, and the number of viable cells were determined in each organ. Prior to killing the mice, urine was collected in a microtube (by pressuring the mouse bladder) for quantitative determination of microorganisms by plating onto CLED agar following a standard technique (Barry *et al.*, 1975). Results are expressed as the mean value of the data obtained from four or five animals.

Histologic procedures

Kidneys from all the groups of mice were fixed in 10% formaldehyde solution for 24 h. The organs were processed and sectioned using to routine procedures. Paraffin sections (5 μm each) were stained with hematoxylin and eosin. Microscopic observation was performed using a light microscope.

Renal function and 2,4-D determinations

Before the animals were killed, they were bled at the retroorbital venous plexus. Serum was obtained for the determination of urea and creatinine by conventional colorimetric methods (Henry *et al.*, 1974).

The 2,4-D levels in kidneys and urine were determined according to the (Yip, 1971) method, with some modifications (Duffard *et al.*, 1987). Briefly, 2,4-D was extracted with solvents and esterified with 5% sulfuric acid in methanol. A Konic gas chromatograph equipped with an electron capture detector with a ^{65}Ni source and a 0.25-inch internal diameter × 6-foot borosilicate glass column, packed with 3% OV-17 on 80/100 mesh chromorsob W-AW, were used. The column temperature was 195 °C, the injection temperature was 250 °C, the detector temperature was 330 °C, and the nitrogen carrier gage pressure was 35 lb in^{-2}. The injection volume was 1 μL. For quantitative measurement, 50 ng of the standard herbicide (methyl ester) was injected. Recoveries of the herbicide standard from organ homogenates that had been fortified prior to extraction were 70%.

Statistical analysis

Data are presented as mean ± SD; statistical differences were assessed by one-way ANOVA followed by the Tukey–Kramer

multiple comparisons test. Levels of significance are shown in the figures; in all cases, the differences were considered significant at $P < 0.05$.

Results

Selection of strains

The predominant phenotypes in the collection strains corresponded to those susceptible to β-lactams (37.5%), strains producing TEM-1-type β-lactamases (35.25%), or strains overproducing TEM-1-type β-lactamases (18.5%). 'TEM-1 type' includes TEM-1, TEM-2 and SHV-1 penicillinases, which were not distinguished on the basis of the resistance pattern. The characterization of the selected strains is summarized in Table 1, by comparing them with a nonpathogenic strain, HB101, and two ATCC standard strains. RM2608 and RM6 isolates showed the same resistance pattern as the penicillinase-overproducing strain ATCC 35218.

Effect of AOAs on *E. coli* susceptibility to different antibiotics

The AOA concentrations were chosen, for the *in vitro* studies, on the basis of the range present in the mammalian urinary tract of experimentally treated or exposed animals (Eiseman, 1984; Koechel *et al.*, 1984; Prince *et al.*, 1986). Also, the additions of AOAs at the concentrations mentioned did not cause inhibition of growth in any of the tested strains (data not shown). The influence of these compounds on the profile of susceptibility to structurally unrelated antibiotics is shown in Table 2. Analysis of the data revealed a 2–8-fold increase of the MICs and/or MBCs when the strains were exposed to the highest concentrations of the compounds, except in the case of rifampicin. The multiple antibiotic resistance increased with each individual compound in a dose-dependent manner, 1 mM being the most effective concentration in all strains tested. On the other

hand, 0.01 mM had no effect in any of the strains evaluated, and 0.1 mM of all the AOAs increased or did not alter the MICs or MBCs, depending on the AOA and antibiotic tested. The TEM-1-type β-lactamase-producing strain RM3518 was not affected by tetracycline; correspondingly, it was the only strain with a high level of resistance to this antibiotic. Interestingly, the MICs and MBCs obtained with the β-lactamase-overproducing strains RM2608 and RM6 represented clinically significant levels of resistance to cephalothin (64–640 mg L^{-1}).

Increases in resistance levels were determined in *E. coli* strains successively treated with 2,4-D over five complete growth cycles, in an attempt to simulate individuals with prolonged exposure. Under these conditions, clinically significant levels of resistance were acquired by β-lactamase-overproducing and β-lactamase-producing strains after successive exposures; MICs of cephalothin were between 256 and 640 mg L^{-1} with 1 mM pretreatments (Table 3).

Influence of AOAs on Omp profile

All hydrophilic but structurally unrelated antibiotics showed diminished bactericidal activity. Simultaneously, an alteration of the permeability barrier of the cells was observed in the porin patterns of the assayed strains (Fig. 1). AOAs dramatically reduced OmpF expression, whereas the expression of other proteins remained unaltered. Omp mobility in SDS-urea gels from strain AW737 and its mutants AW738 (expressing only OmpF) and AW739 (expressing only OmpC) were used to identify *E. coli* porins.

Effect of AOAs on antibiotic treatment *in vivo*

The selected uropathogenic T149 strain was a β-lactamase producer and was susceptible to cephalothin *in vitro*. Once daily, cephalexin, which is available for oral treatment, was administered; it showed the same antibacterial spectrum as cephalothin. The number of bacteria in urine was not influenced by ET or 2,4-D, but CL in combination with

Table 1. β-Lactam resistance phenotypes of *Escherichia coli* strains

Strain*	β-Lactamase activity	Amoxicillin	Amoxicilli–clavulanate	Ticarcillin	Ticarcillin–clavulanate	Cephalothin	Cefoxitin	β-Lactamase phenotype
RM2608	+	R	R	R	R	I	S	TEM-1 type[†]
RM6	+	R	R	R	R	I	S	TEM-1 type[†]
RM3518	+	R	R	R	I	S	S	TEM-1 type
T149	+	R	I	R	I	S	S	TEM-1 type
RM11	−	S	S	S	S	S	S	−
HB101	−	S	S	S	S	S	S	−
ATCC 25922	−	S	S	S	S	S	S	−
ATCC 35218	+	R	R	R	R	I	S	TEM-1 type[†]

*Strains are described in Materials and methods.
[†]Penicillinase overproducer.
R, resistant; S, sensitive; I, intermediate.

Table 2. Effect of aryloxoalcanoic acids on *Escherichia coli* susceptibility to unrelated antibiotics

	MICs (MBCs) in mg L^{-1} for:						
	Trim	Nor	Chlor	Tet	Rif	Ceph	Cefot
Sensitive strains							
RM11 Control	0.50 (2)	0.015 (0.03)	2 (128)	1 (1)	16 (16)	8 (16)	2 (2)
2,4-D 1 mM	**1 (16)**	**0.06 (0.12)**	**4** (128)	**2 (2)**	16 (16)	**32 (32)**	**8 (8)**
2,4-D 0.1 mM	**1 (8)**	0.015 (0.03)	2 (128)	**2 (2)**	16 (16)	8 (16)	**4 (4)**
2,4-D 0.01 mM	0.50 (2)	0.015 (0.03)	2 (128)	1 (1)	16 (16)	8 (16)	2 (2)
Clofíbric acid 1 mM	**1 (16)**	**0.03** (0.03)	**4** (128)	1 **(4)**	16 (16)	8 **(32)**	2 **(4)**
Clofíbric acid 0.1 mM	0.50 (2)	0.015 **(0.06)**	2 (128)	1 (1)	16 (16)	8 (16)	2 (2)
Clofibric acid 0.01 mM	0.50 (2)	0.015 (0.03)	2 (128)	1 (1)	16 (16)	8 (16)	2 (2)
Ethacrynic acid 1 mM	**1 (16)**	**0.03 (0.06)**	**8 (256)**	**2 (8)**	16 (16)	8 **(32)**	**8 (8)**
Ethacrynic acid 0.1 mM	0.50 (2)	0.015 **(0.06)**	2 (128)	**2 (4)**	16 (16)	8 (16)	**4 (4)**
Ethacrynic acid 0.01 mM	0.50 (2)	0.015 (0.03)	2 (128)	1 (1)	16 (16)	8 (16)	2 (2)
HB101 Control	0.25 (0.50)	1 (1)	4 (16)	1 (1)	32 (128)	8 (8)	0.06 (0.06)
2,4-D 1 mM	**0.50** (0.50)	**2 (2)**	**16 (32)**	**2 (2)**	16 (128)	**16 (16)**	0.06 **(0.12)**
2,4-D 0.1 mM	**0.50** (0.50)	1 (1)	**8 (32)**	1 (1)	32 (128)	8 (8)	0.06 (0.06)
2,4-D 0.01 mM	0.25 (0.50)	1 (1)	4 (16)	1 (1)	32 (128)	8 (8)	0.06 (0.06)
Clofíbric acid 1 mM	**0.50 (1)**	**2 (2)**	**8 (64)**	1 **(2)**	32 (128)	**16 (16)**	0.06 (0.12)
Clofíbric acid 0.1 mM	0.25 (0.50)	1 (1)	4 (16)	1 (1)	16 (128)	8 (8)	0.06 (0.06)
Clofibric acid 0.01 mM	0.25 (0.50)	1 (1)	4 (16)	1 (1)	32 (128)	8 (8)	0.06 (0.06)
Ethacrynic acid 1 mM	**2 (2)**	**4 (8)**	**32 (128)**	**2 (2)**	32 (128)	**16 (16)**	0.06 (0.12)
Ethacrynic acid 0.1 mM	**0.50** (0.50)	**2 (2)**	**8 (16)**	1 (1)	16 (128)	8 (8)	0.06 (0.06)
Ethacrynic acid 0.01 mM	0.25 (0.50)	1 (1)	4 (16)	1 (1)	32 (128)	8 (8)	0.06 (0.06)
β-Lactamase-producing* strains							
T149 Control	0.12 (0.25)	0.03 (0.03)	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
2,4-D 1 mM	**0.25 (0.50)**	**0.25 (0.25)**	**8** (128)	**1** (64)	4 (8)	**32 (32)**	0.06 **(0.50)**
2,4-D 0.1 mM	**0.25 (0.50)**	0.03 (0.03)	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
2,4-D 0.01 mM	0.12 (0.25)	0.03 (0.03)	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
Clofíbric acid 1 mM	**0.50 (1)**	**0.12 (0.12)**	**16** (128)	**2** (64)	8 (8)	**16 (32)**	0.06 **(0.12)**
Clofíbric acid 0.1 mM	**0.50 (1)**	**0.06 (0.06)**	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
Clofibric acid 0.01 mM	0.12 (0.25)	0.03 (0.03)	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
Ethacrynic acid 1 mM	**0.50 (2)**	**0.25 (0.50)**	**8 (256)**	**1** (64)	4 (8)	**32 (32)**	0.06 **(0.25)**
Ethacrynic acid 0.1 mM	**0.50 (1)**	**0.12 (0.12)**	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
Ethacrynic acid 0.01 mM	0.12 (0.25)	0.03 (0.03)	4 (128)	0.50 (64)	8 (8)	8 (16)	0.06 (0.06)
RM3518 Control	80 (640)	0.06 (0.06)	8 (16)	160 (320)	8 (8)	8 (8)	0.03 (0.06)
2,4-D 1 mM	**160** (640)	**0.25 (0.25)**	**16 (16)**	160 (320)	8 (8)	**16 (16)**	**0.06 (0.12)**
2,4-D 0.1 mM	80 (640)	0.06 (0.06)	**16 (16)**	160 (320)	8 (8)	**16 (16)**	**0.06 (0.12)**
2,4-D 0.01 mM	80 (640)	0.06 (0.06)	8 (16)	160 (320)	8 (8)	8 (8)	0.03 (0.06)
Clofíbric acid 1 mM	**320** (640)	**0.12 (0.50)**	**16 (64)**	160 (320)	8 (8)	**32 (32)**	**0.12 (0.25)**
Clofíbric acid 0.1 mM	80 (640)	0.06 (0.06)	8 (16)	160 (320)	8 (8)	**16 (16)**	**0.06 (0.12)**
Clofibric acid 0.01 mM	80 (640)	0.06 (0.06)	8 (16)	160 (320)	8 (8)	8 (8)	0.03 (0.06)
Ethacrynic acid 1 mM	**320** (640)	**0.12 (0.25)**	**32 (64)**	160 (320)	Rif (8)	**16 (32)**	**0.06 (0.12)**
Ethacrynic acid 0.1 mM	80 (640)	0.06 (0.06)	**16 (16)**	160 (320)	8 (8)	8 (8)	0.03 (0.06)
Ethacrynic acid 0.01 mM	80 (640)	0.06 (0.06)	8 (16)	160 (320)	8 (8)	8 (8)	0.03 (0.06)
β-Lactamase-overproducing[†] strains							
RM6 Control	0.03 (0.06)	0.06 (0.12)	4 (4)	0.25 (0.25)	8 (16)	40 (80)	0.03 (0.03)
2,4-D 1 mM	0.03 **(0.12)**	**0.12 (0.25)**	**8 (8)**	**0.50 (2)**	8 (16)	**320 (640)**	**0.06 (0.25)**
2,4-D 0.1 mM	0.03 (0.06)	**0.12 (0.25)**	4 **(8)**	0.25 (0.25)	8 (16)	**80** (80)	0.03 (0.03)
2,4-D 0.01 mM	0.03 (0.06)	0.06 (0.12)	4 (4)	0.25 (0.25)	8 (16)	40 (80)	0.03 (0.03)
Clofíbric acid 1 mM	**0.06** (0.06)	**0.12** (0.12)	**16 (16)**	**0.50 (0.50)**	8 (16)	**80** (80)	**0.06 (0.06)**
Clofíbric acid 0.1 mM	0.03 (0.06)	0.06 (0.12)	4 **(8)**	0.25 (0.25)	8 (16)	40 (80)	0.03 (0.03)
Clofibric acid 0.01 mM	0.03 (0.06)	0.06 (0.12)	4 (4)	0.25 (0.25)	8 (16)	40 (80)	0.03 (0.03)
Ethacrynic acid 1 mM	**0.06** (0.06)	**0.12 (0.25)**	**8 (32)**	**0.50 (0.50)**	8 (16)	**80** (80)	**0.06 (0.06)**
Ethacrynic acid 0.1 mM	0.03 (0.06)	0.06 (0.12)	4 **(8)**	0.25 (0.25)	8 (16)	40 (80)	0.03 (0.03)
Ethacrynic acid 0.01 mM	0.03 (0.06)	0.06 (0.12)	4 (4)	0.25 (0.25)	8 (16)	40 (80)	0.03 (0.03)
RM2608 Control	0.12 (0.25)	0.50 (0.50)	8 (8)	2 (8)	16 (16)	64 (128)	0.25 (0.25)
2,4-D 1 mM	**0.25 (0.50)**	**1 (1)**	**16 (32)**	**4 (32)**	16 (16)	**128 (256)**	**0.50 (0.50)**
2,4-D 0.1 mM	**0.25 (0.50)**	0.50 (0.50)	8 (8)	2 (8)	16 (16)	64 (128)	0.25 (0.25)

Table 2. Continued.

	MICs (MBCs) in mg L^{-1} for:						
	Trim	Nor	Chlor	Tet	Rif	Ceph	Cefot
2,4-D 0.01 mM	0.12 (0.25)	0.50 (0.50)	8 (8)	2 (8)	16 (16)	64 (128)	0.25 (0.25)
Clofíbric acid 1 mM	**0.50 (0.50)**	**2 (2)**	**16 (16)**	**4 (16)**	16 (16)	**128 (256)**	**0.50 (0.50)**
Clofíbric acid 0.1 mM	**0.25** (0.25)	0.50 (0.50)	8 (8)	2 (8)	16 (16)	64 (128)	0.25 (0.25)
Clofibric acid 0.01 mM	0.12 (0.25)	0.50 (0.50)	8 (8)	2 (8)	16 (16)	64 (128)	0.25 (0.25)
Ethacrynic acid 1 mM	**0.50 (0.50)**	**2 (2)**	**16 (16)**	**4 (16)**	16 (16)	**128** (128)	**0.50 (0.50)**
Ethacrynic acid 0.1 mM	**0.25** (0.25)	0.50 (0.50)	**16 (16)**	2 (8)	16 (16)	64 (128)	0.25 (0.25)
Ethacrynic acid 0.01 mM	0.12 (0.25)	0.50 (0.50)	8 (8)	2 (8)	16 (16)	64 (128)	0.25 (0.25)

Values represent the mean of three independent experiments; bold type indicates increased values (2–8-fold) relative to control MICs and MBCs.
*β-Lactamase TEM-1-type producers.
†β-Lactamase TEM-1-type overproducers.
Trim, trimethoprim; Nor, norfloxacin; Chlor, chloramphenicol; Tet, tetracycline; Rif, rifampicin; Ceph, cephalothin; Cefot, cefotaxime; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Table 3. Effect of the herbicide on *Escherichia coli* susceptibility to cephalothin after pre-exposures to 2,4-dichlorophenoxyacetic acid (2,4-D); values are MICs (MBCs) in mg L^{-1}

Strains	Control	2,4-D (1 mM)	2,4-D (1 mM) pre-exposed	2,4-D (0.01 mM)	2,4-D (0.01 mM) pre-exposed*
RM2608	64 (128)	128 (256)	**256 (512)**	64 (128)	128 (256)
RM6	40 (80)	**320 (640)**	**640 (1280)**	40 (80)	80 (80)
RM3518	8 (8)	16 (16)	**320 (320)**	8 (8)	**40 (40)**
T149	8 (16)	**32** (32)	**320 (320)**	8 (16)	**40** (40)
ATCC 35218	16 (32)	32 (32)	**256 (512)**	16 (32)	**64** (64)

Values represent the mean of three independent replicates of the experiment; bold type indicates the most significantly increased values (4–40-fold) relative to control MICs and MBCs.
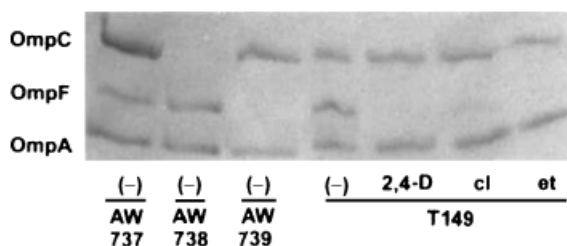*2,4-D pre-exposures in five complete growth curves.

Fig. 1. *Escherichia coli* porin profile in SDS-PAGE plus 8 M urea. Strain AW737 and its isogenic mutants AW738 (OmpF+ OmpC−) and AW739 (OmpF− OmpC+) were grown without treatment (−). Uropathogenic strain T149 was exposed to 2,4-dichlorophenoxyacetic acid, clofibric acid or ethacrynic acid at a concentration of 1 mM.

cephalexin treatment reduced bacteriuria (Fig. 2b). When analyzing the number of *E. coli* CFUs recovered from the kidneys, we observed that the cephalexin treatment was effective. The CFU mean values in the kidneys were between 75% and 89% lower than those in animals without treatment (between 2 and 15 days postchallenge). Notably, ET or 2,4-D annulled the antibiotic therapeutic effect, and, moreover, 2,4-D dramatically increased bacterial colonization after the ninth day of treatment.

To conclude, CL at the therapeutic dose (28.6 mg kg^{-1} day^{-1}) showed no effect on antibiotic treatment of the upper urinary tract, ET at 2.9 mg kg^{-1} day^{-1} neutralized the treatment effects in the kidneys, and 2,4-D at 70 mg kg^{-1} day^{-1} was a therapeutic failure and induced increased bacterial colonization in the kidneys after prolonged exposure (Fig. 2a).

Influence of AOAs and/or antibiotic treatment on renal histology and function

Biochemical parameters associated with renal toxicity, such as blood urea nitrogen and creatinine, were unaltered in our exposure protocol. The antibiotic effect on histology (group II) was a slight swelling of the distal and proximal tubular cells from the fifth day of treatment. CL produced a similar swelling of the proximal tubular cells (Fig. 3d) in groups III and IV, exposed to the hypolipidemic therapy.

In contrast, from the ninth day of exposure, a predominant mononuclear cell infiltrate was observed in the kidneys of mice treated with chlorophenoxyacetic acids (group III mice). This infiltrate was similar in animals treated with ET or 2,4-D and in those belonging to group IV (treated with
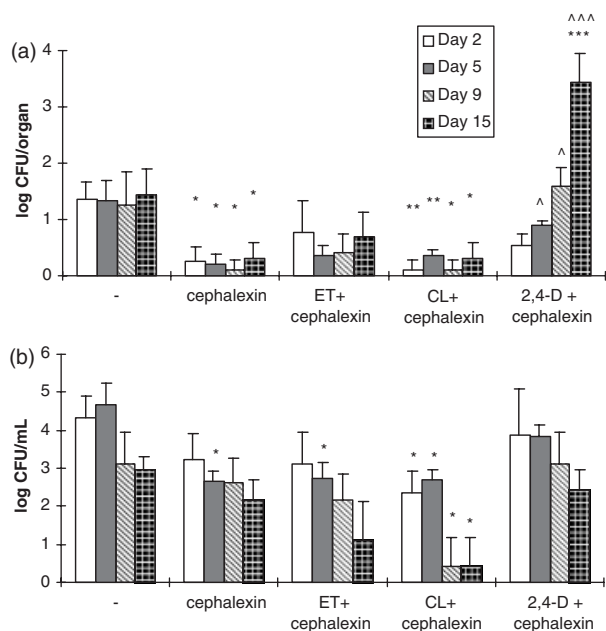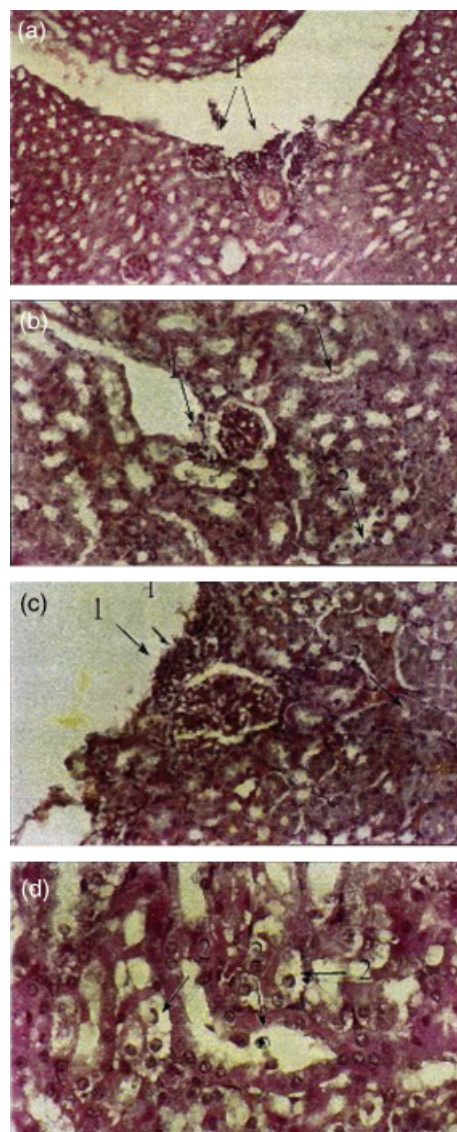
Fig. 2. *Escherichia coli* CFUs recovered from the kidney (a) and urine (b) of experimentally infected BALB/c mice. The day number indicates time after bacterial challenge; the daily oral treatment with drugs was started 1 day after intraurethral bacterial inoculation. Each bar represents the mean+SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ with reference to values for control mice in group I (only challenged with *Escherichia coli*); $\wedge P < 0.05$, $\wedge\wedge\wedge P < 0.001$ with reference to values for mice in the control of treatment group (bacterial challenge and cephalexin treatment); $n = 4$–5.

Fig. 3. Paraffin-embedded sections stained with hematoxylin and eosin demonstrate histologic changes in the kidneys of mice treated with: (a) $70\,mg\,kg^{-1}\,day^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) for 9 days; (b) $70\,mg\,kg^{-1}\,day^{-1}$ 2,4-D for 15 days; (c) $2.9\,mg\,kg^{-1}\,day^{-1}$ ethacrynic acid (ET) for 15 days; and (d) $28.6\,mg\,kg^{-1}\,day^{-1}$ clofibric acid (CL) for 15 days. 1, inflammatory cell infiltrate; 2, alterations of the tubular cells. (a) $\times 100$; (b) and (c) $\times 200$; (d) $\times 400$.

drugs and challenged with *E. coli*), suggesting an induced inflammatory response to these compounds (Fig. 3a–c). In addition, more extensive adverse effects provoked by 2,4-D were observed after prolonged exposure, such as tubular acidosis, cellular desquamation and detritus in tubules in the last treatment period (Fig. 3b). Tubular acidosis was related to colorless tubular cells without nuclei or with protruding nuclei.

Concentrations of 2,4-D measured during this last period were higher in urine than in the kidneys. Measurements made in the period 4–12 h after the oral dose gave values in the range 0.058–0.290 mM in urine and in the range 0.012–0.062 mM in kidney homogenates, representing an *c.* fivefold difference.

Discussion

The effects of weak AOAs on *in vitro* susceptibility and on the experimental treatment of UTI caused by uropathogenic *E. coli* were analyzed in this work. We chose three compounds that were structurally related in containing a chlorinated phenoxy moiety. The loop diuretic ET and the selective herbicide 2,4-D are agents derived from phenoxyacetic acid, whereas the prototypical hypolipidemic drug

CL is derived from phenoxypropionic acid. These compounds are mainly excreted by the renal route; CL is excreted in part as a glucuronide conjugate; 2,4-D and two-thirds of the dose of ET are secreted essentially unaltered in urine by the organic anion transport system (Erne, 1966; Taskar *et al.*, 1982; Knopp & Glass, 1991; Jackson, 1996; Witztum, 1996). This means that in the urinary tract of exposed or treated individuals, pathogenic microorganisms will also be exposed to these drugs.

To analyze the potential influence of AOAs, we determined their effects on the antibiotic susceptibility of uropathogenic *E. coli* isolates selected on the basis of the pattern of their resistance to β-lactams. We chose five strains representing the predominant phenotypes found in our collection: susceptible strains, and strains producing or overproducing TEM-1-type β-lactamases. In addition, we chose hydrophilic antibiotics with different mechanisms of action and a hydrophobic drug (rifampicin). All the tested compounds caused loss of susceptibility in all strains and with all the antimicrobial drugs, giving increases in the MICs and/or MBCs from two-fold to eight-fold, with the exception of rifampicin. These results suggest that the nonspecific hydrophilic pathways of the outer membrane could be involved. Relatively hydrophilic drugs can readily penetrate through the porin channels of enteric bacteria. In *E. coli*, two of these outer membrane pores, OmpC and OmpF, function as hydrophilic diffusion channels, but the rate of diffusion through OmpF is *c.* 10 times greater than the rate through OmpC (Nikaido, 1996). Salicylate (an aromatic weak acid that is produced in plants in response to invasion by microorganisms) is known to repress the synthesis of OmpF and to make *E. coli* more resistant to chloramphenicol, tetracycline, quinolones and ampicillin (Rosner, 1985). This resistance induction may occur via several pathways, including the weak acid-inducible and antibiotic-inducible *marRAB* operon (Rosner & Slonczewski, 1994; Balagué & Véscovi, 2001). Accordingly, the Omp profile of bacteria treated with AOAs showed a remarkable diminution of OmpF (the most effective hydrophilic channel expressed in *E. coli*).

Mutants with decreased expression of porins are not very resistant to certain antibiotics, but significant levels of drug resistance are observed when other resistance mechanisms are present, such as active efflux (OmpF resistance is closely linked with induction of efflux pumps) or β-lactamase production (Nikaido, 1994). Our results consistently showed that strains overproducing TEM-1-type β-lactamases achieved clinically significant levels of resistance to cephalothin, a drug rapidly hydrolyzed by the enzyme. Interestingly, when strains were successively exposed to 2,4-D, even the strains producing the enzyme showed very high resistance levels (4–40-fold in relation to control MICs and MBCs). Moreover, pre-exposure was able to induce resistance, even with the low concentration detected in kidney homogenates (0.01 mM). In contrast, cefotaxime is a β-lactam antibiotic that is more resistant to hydrolysis by TEM-1-type β-lactamases; therefore, significant levels of resistance to cefotaxime were not observed.

To assess the influence of AOAs in the *in vivo* treatment of UTI, we used the murine model of ascending infection previously described (Nader-Macías *et al.*, 1996; Silva de Ruiz *et al.*, 1996). Therapeutic doses of the pharmacologic

agents were chosen, and the 2,4-D exposure dose was selected on the basis of our previous work (Balagué *et al.*, 2002).

The number of bacterial CFUs recovered in kidneys clearly demonstrated a therapeutic antibiotic failure due to 2,4-D exposure, and ET annulled the effect of the treatment. These phenoxyacetic acids would affect cephalexin pharmacokinetics (both secretion and reabsorption) and promote excretion of the antibiotic in urine or retention in blood. Other inhibitors of organic anion transport show biphasic effects on pharmacokinetics, with promotion of retention at low doses of the inhibitor, and promotion of excretion at high doses (Jackson, 1996), affecting *in vivo* results. The phenoxypropionic acid CL did not show the same effect, even though it was administered at a dose 10-fold higher than that of ET. These results suggest that the dissimilar effect of CL could be attributed to its chemical structure being less related to that of phenoxyacetic acids. Moreover, perhaps a more important difference, in terms of biological activity, among the three compounds concerns the number and position of the chlorine groups on the phenyl ring. CL has only one chlorine group at the 4-position, whereas ET and 2,4-D have two chlorine groups at the 2,3-position and 2,4-position, respectively. As the pharmacokinetics of the hypolipidemic drug also differ from those of 2,4-D and ET, only *in vivo* assays in the murine model would reveal the different influences of AOAs on therapeutics. It is apparent that the glucuronide conjugate fraction of CL excreted in urine was ineffective in inducing resistance to cephalexin.

In addition, 2,4-D-treated mice showed dramatically high renal colonization after prolonged exposure. This result led us to investigate renal toxicity parameters and histology. No changes in serum urea or creatinine values, in comparison to control mice, were detected. Several other authors have found no alterations in renal function with up to 100 mg kg^{-1} 2,4-D or 100 mg kg^{-1} 2,4,5-trichlorophenoxyacetic acid exposure in rats (Koschier & Hong, 1981; Kukowicz-Ratajczak & Krechniak, 1988).

ET and 2,4-D are agents actively secreted by the renal proximal tubular cells. These drugs might accumulate in tubular cells, and therefore may have detrimental effects on the kidneys due to an increase in their concentrations in the cells relative to blood. Tubular cell vacuolation was observed in dogs treated with elevated doses of ET (Koechel *et al.*, 1984). We observed tubular shedding with therapeutic doses after prolonged treatment in our mouse experimental model. Similar but more detrimental effects on renal proximal tubular cells were caused by 2,4-D; acidosis, cellular desquamation and detritus in the tubules were observed in the last period of treatment. Even though the 2,4-D concentration in kidney was fivefold lower than that in urine, tubular damage was detected in each animal examined. These results revealed the high sensitivity of renal

tissue to the herbicide. This aspect was correlated with extremely high bacterial colonization in the kidney, suggesting greater *E. coli* invasiveness in this condition. We suggest that the increased CFU number detected in kidney after the first 9 days of 70 mg kg^{-1} day^{-1} exposure could be related to 2,4-D-saturated renal clearance and tissue damage.

There were different histologic changes in the kidney after prolonged treatment with CL and the antibiotic; swelling of the proximal tubular cells was only observed in groups II, III (CL) and IV (CL).

In our study, no additive nephrotoxic effect was seen with cephalexin and each AOA. Similar tubular alterations were observed in animals treated with 2,4-D, CL or ET and in those exposed to each AOA and simultaneously treated with the antibiotic.

This study showed the induction of antibiotic resistance (*in vitro* and *in vivo*) by compounds that are used pharmacologically and by a chemical herbicide that is widely used. The results reinforce the notion that the impact of chemical exposure is frequently underestimated or ignored when an antibiotic therapy is established.

Acknowledgements

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