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## Article

# In-Vitro Activity of N,N-Diethyldithiocarbamate against *Ureaplasma urealyticum* and *Ureaplasma parvum* Isolated from Women with Infections of the Urogenital Tract

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**Abstract:** *Ureaplasma* species (*Ureaplasma* spp.) are commonly found as commensals in the human urogenital tracts, but their overgrowth can lead to infection in the urogenital tract and at distal sites. Treatment of *Ureaplasma* spp. infections is difficult. The lack of a cell wall confers resistance to all  $\beta$ -lactam and glycopeptide antibiotics whereas the lack of *de novo* synthesis of folic acid makes them resistant to sulfonamides and diaminopyrimidines. Thus, there is a need to search for new drugs effective against these bacteria. In the presented studies, the effect of N,N-diethyldithiocarbamate (DDC) at a concentration of 2  $\mu$ g/ml, 20  $\mu$ g/ml and 200  $\mu$ g/ml on the growth and survival of *Ureaplasma urealyticum* and *Ureaplasma parvum* was tested. Our results indicated that all DDC concentrations used showed both bacteriostatic and bactericidal activity against both tested strains. In brief, for the first time we showed the bacteriostatic and bactericidal activity of DDC against *Ureaplasma urealyticum* and *Ureaplasma parvum*. Based on literature data, we conclude that this DDC activity is related to the inhibition of urease activity, which is an indispensable enzyme for these bacteria. Further research is needed and the results obtained in the present study should be verified in *in vivo* models of infection.

**Keywords:** *Ureaplasma urealyticum*; *Ureaplasma parvum*; N,N-diethyldithiocarbamate; disulfiram; urease

## 1. Introduction

The *Ureaplasma* is the genus of bacteria belonging to the family *Mycoplasmataceae* in the order *Mycoplasmatales*. Members of the family *Mycoplasmataceae* include small pleomorphic bacteria that characteristically lack a cell wall. The genus *Ureaplasma* is biochemically unique in that all its members possess urease and therefore hydrolyze urea to produce ATP. *Ureaplasma* species (*Ureaplasma* spp.) are commonly found as commensals in the human urogenital tracts but their overgrowth can lead to infection in the urogenital tract and, rarely, at distal sites [1,2]. *Ureaplasma urealyticum* (*U. urealyticum*) and *Ureaplasma parvum* (*U. parvum*) formerly considered as two distinct biovars of *U. urealyticum*, are now classified as separate species and are the only ureaplasmas associated with disease in humans. *U. urealyticum* was first isolated in men with non-gonococcal urethritis (NGU) in 1954 [3]. Some authors have reported that *U. urealyticum* strains are detected more often in men with NGU than in men without NGU [4-6]. It has also been demonstrated that *U. parvum* strains are found more often than *U. urealyticum* strains in men without NGU [7,8]. It has also been shown that *U. urealyticum* may be the causative agent in some cases of chronic prostatitis [9].

However, due to the anatomical differences in the structure of the urogenital tract of women and men *Ureaplasma* spp. are more often identified in women than in men. Epidemiological data indicate that nearly 90% of sexually active healthy women are carriers of *Ureaplasma* spp. [10]. As already mentioned, *Ureaplasma* spp. are generally regarded as low-virulent commensals. It is known,

however, that colonization of the genital tract with *Ureaplasma* spp. increases especially in the third trimester of pregnancy. Intraamniotic *Ureaplasma* spp. infections increase the risk for chorioamnionitis, premature rupture of membranes, and preterm birth. Moreover, these bacteria may be transmitted from mothers to neonates, either *in utero* or during passage through the infected birth canal. Vertical transmission rates are maximal among preterm infants with very low birthweight (<1500g) [11,12]. These microorganisms mainly colonize the respiratory tract of newborns, and their presence can cause numerous life-threatening disorders. There is no clear difference in the pathogenicity of both species of ureaplasmas. According to Katz et al. and Heggie et al. risk of development of bronchopulmonary dysplasia in neonates infected by *U. parvum* and *U. urealyticum* is similar [13,14]. However, other authors assess that the chance of developing bronchopulmonary dysplasia in children colonized by *U. urealyticum* is 56%, and in the case of *U. parvum* it reaches 25% [15].

Moreover, it has been shown that the presence of *U. urealyticum* in the mother's reproductive tract affects also the central nervous system (CNS) of neonates and causes meningitis, intraventricular hemorrhage and hydrocephalus [16]. In addition, there is strong evidence from experimental animal studies and clinical trials indicating that genital ureaplasma infections cause female infertility, mainly because these bacteria can enter the upper genital tract and colonize the endometrium, fallopian tubes and ovaries. This condition known as pelvic inflammatory disease has serious long-term consequences, including infertility [17]. This problem also applies to men. It has been indicated that *U. urealyticum* produces a toxic factor that impairs sperm function [18,19].

Treatment of *Ureaplasma* infections is exceptionally difficult. The lack of a cell wall confers resistance to all  $\beta$ -lactam and glycopeptide antibiotics whereas the lack of *de novo* synthesis of folic acid makes them resistant to sulfonamides and diaminopyrimidines. Based on current knowledge, only four classes of antibiotics are considered effective in treating *Ureaplasma* infections. These are the antibiotics belonging to the fluoroquinolone, tetracycline, chloramphenicol and macrolide classes [20]. However, the management of *Ureaplasma* infections is possible only when dealing with antibiotic-susceptible strains. However, many authors point to the growing incidence of antibiotic-resistant strains of *Ureaplasma* in the population. Research by Khosropour et al. indicated that 57% of individuals with NGU who were infected with *Ureaplasma* spp. and received antimicrobial therapy with initially azithromycin followed by doxycycline for 7 days, or vice versa, remained still colonized with these bacteria after 6 weeks of therapy [21]. Biernat-Sudolska et al. showed that as many as 90% of *U. parvum* and 83% of *U. urealyticum* strains present in endotracheal aspirate from prematurely born neonates were resistant to ciprofloxacin belonging to the fluoroquinolone class [22].

Thus, there is a need to search for new drugs effective against these atypical bacteria. We chose N,N-diethyldithiocarbamate (DDC) for our research. DDC is the main metabolite of disulfiram (DSF) that is an irreversible inhibitor of aldehyde dehydrogenases (ALDHs), enzymes that catalyze the oxidation of aldehydes to carboxylic acids. For this reason, DSF has been used for over 70 years in the treatment of alcohol addiction. However, it is increasingly believed that the use of DSF as a treatment in patients with alcoholism is an unethical therapy because it is connected with the risk of poisoning and even a loss of life. Thus, at present the use of DSF in the treatment of alcoholism has declined. However, there have been reports indicating new areas of pharmacological activity of DSF and its metabolites. For this reason, DSF has been studied as a possible treatment for cancer, and viral, bacterial, parasitic and fungal infections [23-27].

In this study, we examined for the first time the bacteriostatic (effects on the cell division) and bactericidal activity of DDC against *U. urealyticum* and *U. parvum*.

## 2. Material and Methods

### 2.1. General

*Ureaplasma* strains isolated in the Chair of Microbiology of the Jagiellonian University Medical College in Kraków (Poland) from women with infections of the urogenital tract were tested in the

study. The study involved 90 strains of *Ureaplasma* spp. including 36 *U. urealyticum* strains and 54 *U. parvum* strains.

## 2.2. Detection and identification of *Ureaplasma* spp

The vaginal swabs were collected from each woman and placed in BioMerieux transport media. Next, they were subcultured in liquid and solid PPLO media with 1% urea, prepared in-house according to the previously described procedure [Shepard 1986] and parallelly in Mycoplasma IST 2 kit BioMerieux medium (R2). Identification of ureaplasma species was done by PCR using the previously described two pairs of primers specific for the *U. urealyticum* and *U. parvum* genes [28]. Reference strains: *U. urealyticum* (ATCC 27816) and *U. parvum* (ATCC 27815) were used as the positive control. Negative control contained of distilled water instead of bacteria. The amplified products were visualized under ultraviolet light after agarose gel electrophoresis containing ethidium bromide (EtBr).

## 2.3. The effect of DDC on *Ureaplasma* spp

Baseline titers (as a control) were established for each strain by titration of an 18-24 hour culture in growth medium without the addition of DDC. The titer for each *Ureaplasma* strain was estimated using the color changing units (CCU) assay. Titration was performed in 96-well plates using a series of 10-fold dilutions ( $10^{-1}$  to  $10^{-10}$ ). The titer was read after 24-hour incubation at 37°C. The CCU is defined as the highest dilution at which there is a color change [29]. In parallel, the effect of DDC at a concentration of 2 µg/ml, 20 µg/ml and 200 µg/ml on the growth and survival of *Ureaplasma* strains was tested. The highest concentration of 200 µg/ml used in the studies was also the highest non-toxic concentration of DDC for cell culture of the RK13 line, which was previously determined in *in vitro* studies.

## 2.4. Bacteriostatic effect of DDC on *Ureaplasma* cultures

The cultures of each *Ureaplasma* strain were titrated by making 10-fold serial dilutions ( $10^{-1}$  to  $10^{-10}$ ) in growth medium with the addition of the tested DDC concentrations. Titration was carried out in 96-well plates. The titers were read after 24 hours of incubation at 37°C and expressed as CCU/ml. The obtained values were compared with the control titer for a given strain.

## 2.5. Bactericidal activity of DDC

The culture of each *Ureaplasma* strain was suspended in medium with the tested concentrations of DDC and was incubated for 24 hours at 37°C. After incubation, the culture was centrifuged (Sigma 113 microcentrifuge; 14,000G/20,000 rpm) and washed twice with saline to remove DDC and then titration was performed by making 10-fold serial dilutions ( $10^{-1}$  to  $10^{-10}$ ) with growth medium without the addition of DDC similarly as described above. The obtained values were compared with the control titer for a given strain.

## 2.6. Statistical analysis

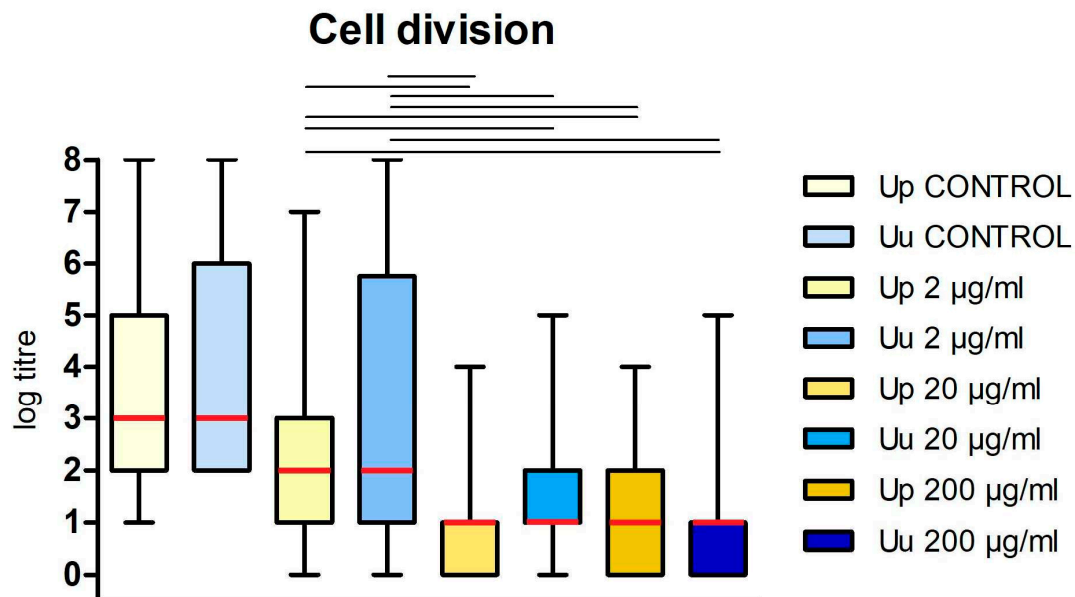
Statistical calculations were carried out with the IBM® SPSS® Statistic 29 (Armonk, NY: IBM Corp, USA). Differences between the study groups were analyzed using Kruskal-Wallis non-parametric tests with Dunn's post-hoc test. Statistical significance was defined as  $p < 0.05$  for all tests.

# 3. Results

## 3.1. Bacteriostatic effect of DDC on *Ureaplasma* cultures

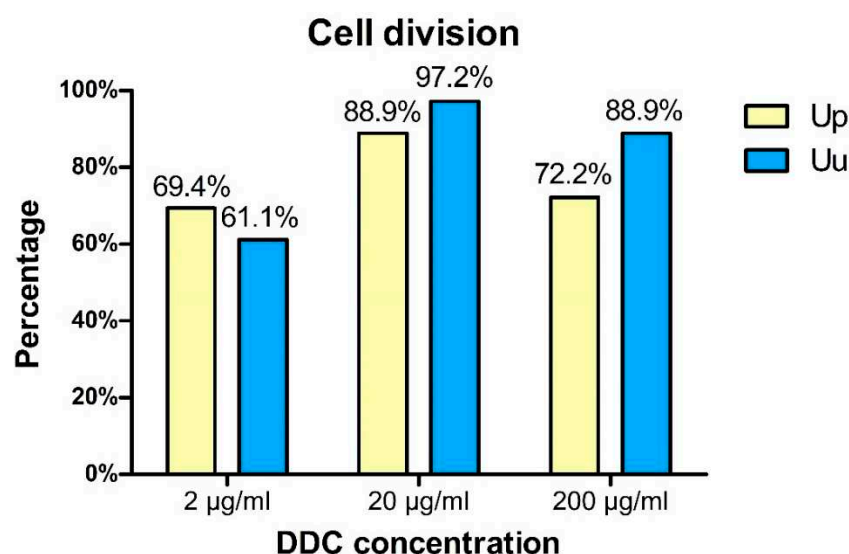
The median value of log titre for control *U. parvum* cultures was 3 (2, 5) while the median values of log titre for *U. parvum* cultured in the presence of DDC at a concentration of 200 µg/ml, 20 µg/ml, and 2 µg/ml were 1 (0, 2), 1 (0, 1) and 2 (1, 3) respectively. The differences between *U. parvum* titers obtained in the control system (without DDC) and the tested system (in the presence of DDC) were

statistically significant ( $p < 0.001$ ) (Figure 1). The median value of log titre for control *U. urealyticum* cultures was 3 (2, 6) while the median values of log titre for *U. urealyticum* cultured in the presence of DDC at a concentration of 200  $\mu\text{g/ml}$ , 20  $\mu\text{g/ml}$ , and 2  $\mu\text{g/ml}$  were 1 (0, 1), 1 (1, 2) and 2 (1, 5.75), respectively. The differences between *U. urealyticum* titers obtained in the control system (without DDC) and the tested system (in the presence of DDC) were statistically significant ( $p < 0.05$ ) (Figure 1). Therefore, the obtained results showed that the presence of DDC affected the multiplication rate (cell division) of both tested strains.



**Figure 1.** The bacteriostatic effect of DDC on the cells of *Ureaplasma* spp. The graph shows the dependence of log titre for *U. parvum* (Up) and *U. urealyticum* (Uu) on DDC dose. Data are shown as the median value (red rectangle) with upper and lower quartyl. The whiskers represent the minimum and maximum values. Groups connected by a line are statistically significant at  $p < 0.05$ .

The obtained results demonstrated that strains of the *U. urealyticum* are more sensitive to the bacteriostatic effect of DDC at doses of 20 and 200  $\mu\text{g/ml}$  than *U. parvum* strains. Only the lowest (2  $\mu\text{g/ml}$ ) used concentration of DDC inhibited the cell division of a larger percentage of strains from the *U. parvum* species than from the *U. urealyticum* species. The bacteriostatic effect of DDC at a dose of 20  $\mu\text{g/ml}$  was observed for 88.9% of *U. parvum* and 97.2% of *U. urealyticum* strains. It means that DDC at a dose of 20  $\mu\text{g/ml}$  elicited the most effective bacteriostatic effect against both tested strains (Figure 2).

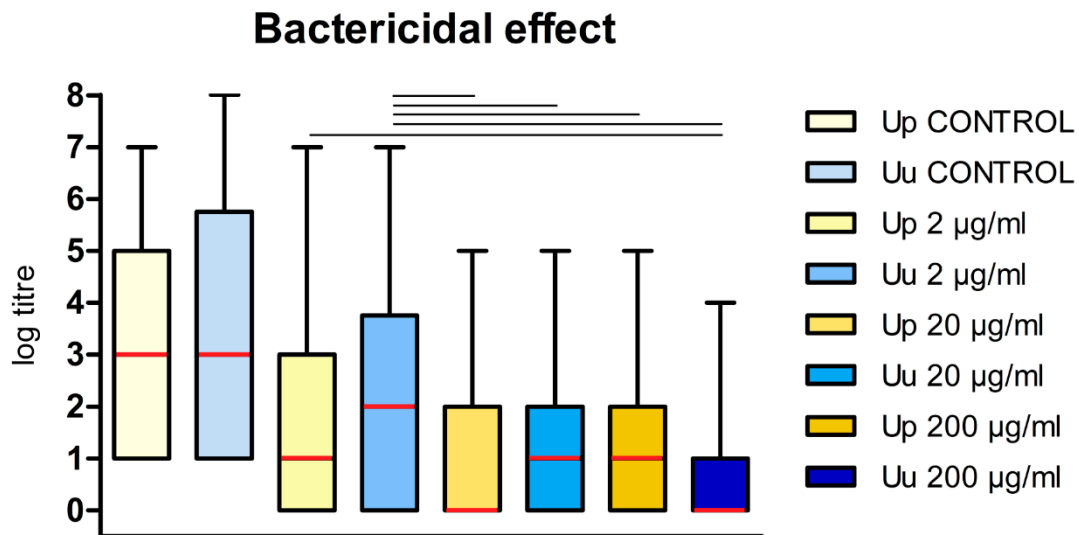




**Figure 2.** Percentage of *U. parvum* (Up) and *U. urealyticum* (Uu) strains for which inhibition of cell division (bacteriostatic activity) was found at different DDC concentrations.

### 3.2. Bactericidal activity of DDC on *Ureaplasma* cultures

The median value of log titre for control *U. parvum* cultures was 3 (1, 5) while the mean values of log titre for *U. parvum* cultured in the presence of DDC at a concentration of 200 µg/ml, 20 µg/ml, and 2 µg/ml were 0 (0, 2), 0 (0, 2) and 1 (0, 3), respectively (Figure 3). The median value of log titre for control *U. urealyticum* cultures was 3 (1, 5.75) while the median values of log titre for *U. urealyticum* cultured in the presence of DDC at a concentration of 200 µg/ml, 20 µg/ml, and 2 µg/ml were 0 (0, 1), 1 (0, 2) and 2 (0, 3.75), respectively (Figure 3).

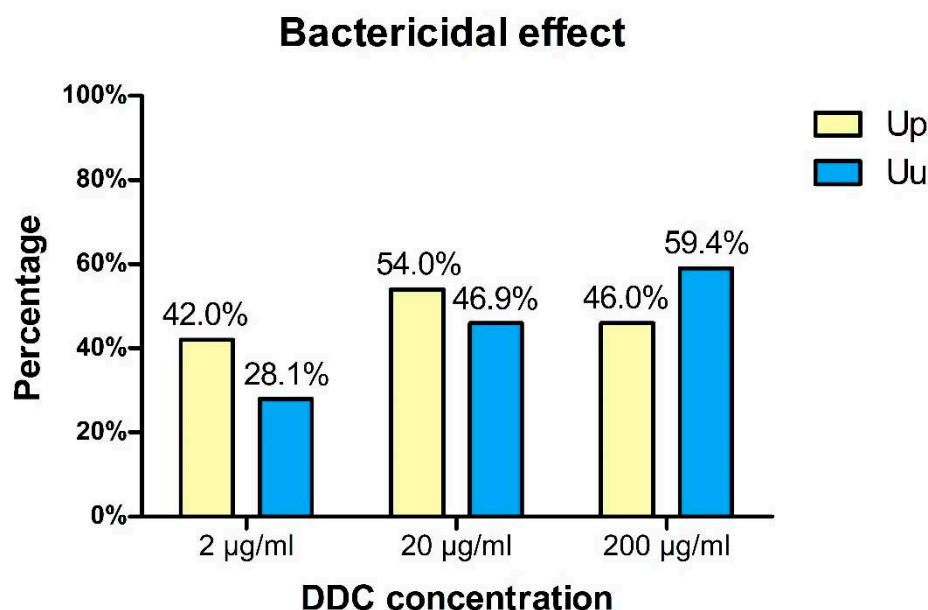


**Figure 3.** The bactericidal effect of DDC on the cells of *Ureaplasma* spp. The graph shows the dependence of log titre for *U. parvum* (Up) and *U. urealyticum* (Uu) on DDC dose. Data are shown as the median value (red rectangle) with upper and lower quartyl. The whiskers represent the minimum and maximum values. Groups connected by a line are statistically significantly different at  $p < 0.05$  level.

It should be noted that although the decrease in bacterial titre correlates with the decrease in bacterial numbers, it does not clearly indicate the bactericidal properties of DDC. In order to test whether DDC has such properties, we conducted an experiment to check whether the tested strains cultured in the presence of DDC retained the ability to grow in growth medium without the addition of DDC. The percentage of strains against which DDC bactericidal activity has been demonstrated is shown in Figure 4.

The obtained results indicated that DDC at a dose of 200 µg/ml had a bactericidal effect against 46% of *U. parvum* strains and 59.4% of *U. urealyticum* strains. After incubation on the medium with DDC at a dose of 20 µg/ml and then transfer of the bacteria to the medium without DDC, no growth was observed for 54% of *U. parvum* strains and 46.9% of *U. urealyticum* strains. DDC at the lowest dose of 2 µg/ml has a bactericidal effect on 42 of *U. parvum* strains and 28.1% of *U. urealyticum* strains.

Thus, the greatest bactericidal effect for *U. urealyticum* strains was observed at a DDC concentration of 20 µg/ml, and for *U. parvum* strains at 200 µg/ml.

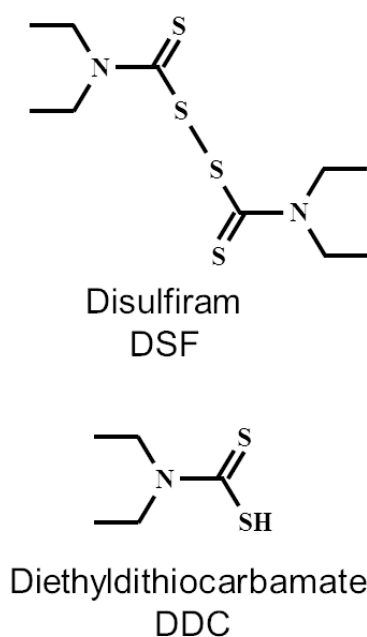


**Figure 4.** Percentage of *U. parvum* (Up) and *U. urealyticum* (Uu) strains for which bactericidal effects of DDC were demonstrated.

#### 4. Discussion

It is known that DDC is the major breakdown product of DSF *in vivo*. One molecule of DSF gives rise to two molecules of DDC by the reduction of intramolecular disulfide bond. In the blood of humans and other animals, DSF is rapidly and completely converted to two molecules of DDC by serum albumin and erythrocyte enzymes [30,31].

So, DDC is the main metabolite of DSF and is generally believed to be responsible for most of the observed pharmacological effects of DSF. The chemical structure of DSF and DDC is shown in Figure 5.



**Figure 5.** Chemical structures of disulfiram and diethyldithiocarbamate.

Our results indicated that all DDC concentrations used in these experiments showed both bacteriostatic and bactericidal activity against both tested *Ureaplasma* strains. The antimicrobial effect of DSF and its main metabolite DDC has been known for a long time. In 1974 Marshall et al. indicated that DDC had activity against *Malassezia pachydermatis* (formerly known as *Pityrosporum canis*) that has been found to cause skin and ear infections, most often occurring in canines. The same authors showed that in addition to having antifungal activity, DDC killed ear mites (*Otodectes cynotis*) in cats [32]. It is worth recalling that otodectosis is the most commonly diagnosed ectoparasitic disease in cats. In 1979 Scheibel et al. showed that DSF inhibited *in vitro* the growth of the human malaria parasite *Plasmodium falciparum* [33].

The studies conducted in the 21st century confirm the antifungal and antiparasitic potential of DSF and DDC. Khan et al. indicated that DSF showed fungicidal activity on *Aspergillus fumigatus* (*A. fumigatus*), *Aspergillus flavus* (*A. flavus*) and *Aspergillus niger* (*A. niger*) [27]. It has also been indicated that DDC showed high antifungal activity against *Candida albicans* (*C. albicans*) and *Candida tropicalis* (*C. tropicalis*) biofilms [34]. Khouri et al. demonstrated that DDC dose-dependently induced parasite killing and was able to "sterilize" *Leishmania amazonensis* (*L. amazonensis*) infection at 2 mM in human macrophage *in vitro* cultures [35]. Celes et al. tested a topical formulation of bacterial cellulose (BC) membranes containing DDC. The obtained results indicated that exposure of *Leishmania braziliensis* (*L. braziliensis*)-infected cells to BC-DDC decreased the parasite load in a significant manner [36]. In 1987 Taylor et al. demonstrated that DDC inhibited the growth of methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) *in vitro* [37]. Later research showed that DSF also inhibited the growth of MRSA [38]. Multidrug-resistant (MDR) *S. aureus* is associated with high rates of serious disease and treatment failures. It has recently been shown that DSF *in vitro* potentiates vancomycin (VAN) susceptibility in vancomycin-resistant *S. aureus* (VRSA) strains [39]. It has also recently been shown that the combination of DDC and copper ions is active against *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) biofilms *in vitro* and *in vivo* [40]. Kobatake et al. recently showed that DSF had a bactericidal effect on *Helicobacter pylori* (*H. pylori*) at a low concentration (1 µg/mL) suggesting that it can be used as a supplement to current *H. pylori* eradication drugs. Moreover, the authors showed that DSF reduced the expression levels of proteins, such as urease, VacA (vacuolating cytotoxin) and CagA (cytotoxin-associated gene A) proteins that are the virulence proteins of *H. pylori* [41].

Thus, the position of DSF and its metabolites as antibiotics should not be contested. Unfortunately, most studies on the antibacterial properties of DSF and its metabolites focus on various Gram-positive and Gram-negative pathogens, with little attention to the genus *Ureaplasma*. In various bibliographic databases we were able to find only one paper on the effect of DSF on bacteria from the *Mollicutes* class which also includes the genus *Ureaplasma*. The results obtained by the authors showed that DSF exhibited copper-dependent antimicrobial activity against *Mycoplasma hominis* (*M. hominis*) [42].

In our previous research on bacteria from the *Mollicutes* class, we observed that lipoic acid (LA) had a statistically significant inhibitory effect on cell division of *U. urealyticum* and *U. parvum* cells *in vitro* compared to the control [43], while it did not affect the growth of *M. hominis* cells *in vitro* [44].

We recall these studies because LA, like DSF, is characterized by the presence of disulfide bonds. However, dihydrolipoic acid (DHLA), which is a reduced form of LA, is a thiol, like DDC. So, both LA and DSF are electrophiles that readily form disulfides with thiol-bearing substances. Bacteria contain many intracellular compounds containing thiophilic residues (for example cysteine (Cys), coenzyme A (CoA), glutathione (GSH), thioredoxin (Trx)) can be potentially modified by LA and DSF via thiol-disulfide exchange, which can lead to antimicrobial effects [39]. In addition, the reaction of intracellular thiols with DSF results in the generation of DDC that have metal (e.g. copper, iron, zinc)-chelating properties. As a result, DDC is able to inhibit the activity of metalloenzymes in the bacterial cells [45]. On the other hand, DDC or DHLA that are thiol-bearing substances can potentially modify compounds containing disulfide bonds present in the bacterial cell, for example oxidized glutathione (GSSG) or oxidized cystine (Cys-S-S-Cys) by thiol-disulfide exchange. „The Thiol World is an extremely interesting and challenging world from which many mysteries are still to be discovered” [46]. The thiol-disulfide exchange is especially expected to occur with cellular GSH/GSSG. This causes



a decrease in GSH levels in bacterial cells which can lead to antimicrobial effects. In studies on *S.aureus*, Long showed that the introduction of GSH in the growth medium caused an increase in the minimum inhibitory concentrations in µg/mL (MICs) of DSF and DDC [39]. In our opinion, the most likely mechanism by which DDC produces a bactericidal and bacteriostatic effect on *U. urealyticum* and *U. parvum* cells is related to the inhibition of urease (urea amidohydrolase, EC 3.5.1.5) activity. It is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form carbonic acid and two molecules of ammonia. Urease activity has been detected in a large variety of bacteria but the ureaplasmas are the only organisms known to depend on urea for growth [47]. *Ureaplasmas* lack the conventional mechanisms for ATP generation, such as glycolysis or arginine breakdown, present in other mycoplasmas. It has been indicated that ATP is generated in these organisms through the formation of an ion gradient coupled to urea hydrolysis that is catalyzed by urease [48,49,50]. Thiol compounds are urease inhibitors, which was first demonstrated in 1980. The authors indicated then that β-mercaptoethanol (β-ME) acted as a competitive inhibitor of jack bean urease (JBU) [51]. Benini et al. demonstrated that β-ME inhibited *Bacillus pasteurii* (*B. pasteurii*) urease by bridging the two nickel ions in the enzyme active site through the sulfur atom and chelation one Ni through the OH functionality. The authors also showed that β-ME was involved in a mixed disulfide bond with Cys<sup>α322</sup>, a residue which plays a significant role in the urease-catalyzed process of urea hydrolysis [52]. There have been no studies on the effect of thiol compounds, including DDC on urease activity in *Ureaplasma* genus cells, so far. However, it is known that urease is very highly conserved in sequence and three-dimensional structure [53,54]. So, it can be assumed that the mechanism of the bacteriostatic and bactericidal effect of DDC against *U. urealyticum* and *U. parvum*, proven in the presented studies, is related to the inhibition of urease activity, which is an essential enzyme for these bacteria. In our opinion, it is a plausible proposal.

The problem we investigated certainly requires more research, but it is worth considering DDC as a drug in the treatment of patients with infections caused by *Ureaplasma* genus. An additional advantage of DDC is a lack of teratogenic, mutagenic or carcinogenic effects in animal models [55].

## 5. Conclusions

The clinical consequences of *Ureaplasma* infections can be serious and their treatment is often difficult. Thus, there is a need to search for new drugs effective against these atypical bacteria.

In this study, we showed for the first time the bacteriostatic (effects on the cell division) and bactericidal activity of DDC against *U. urealyticum* and *U. parvum*. Based on literature studies, we conclude that this DDC activity is related to the inhibition of urease activity, which is an essential enzyme for these bacteria. An additional advantage of DDC is a lack of teratogenic, mutagenic or carcinogenic effects in animal models.

In conclusion, DDC showed considerable antimicrobial activity against *U. urealyticum* and *U. parvum* in *in vitro* cultures. Further research is needed and the results obtained in the present study should be verified in *in vivo* models of infection.

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**Conflicts of Interest:** The authors declare that there are no conflicts of interest.

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