

Antibacterial Effects of Novel Thiazole Derivatives and the Toxicity of Oxothiazole on Liver Histopathology of Mice

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ABSTRACT

Background and Objective: Antibiotic resistance in bacteria has actuated researchers toward evaluating many new antibacterial compounds of which are the thiazoles. In this research the inhibitory effects of novel thiazole derivatives were unraveled on *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and oxothiazole liver toxicity effects were assessed on mice.

Methods: The antibacterial effect of thiazole derivatives was evaluated by measuring the halo zone with disk diffusion method and dilution procedure in microplate in order to discriminate the minimum inhibitory concentration (MIC) and the liver toxicity of oxothiazole, also, was discerned by injecting 160 mg/kg, 265 mg/ kg and 350 mg/kg doses to mice as well as scrutinizing the liver histopathology.

Results: Derivatives utilized in experiment had no inhibitory effect on *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, though their inhibitory effect was observed on *Staphylococcus aureus* and *Streptococcus agalactiae*. For *Staphylococcus aureus* and *Streptococcus agalactiae* the diameters of growth inhibition zone were 8.9-22.3 mm and 16.1-25.6 mm, respectively and MIC of 50-200 and 25-100 µg/ml by order. Additionally, by increasing the injection dose of oxothiazole with 160 mg/ml, 265 mg/ml and 350 mg/ml doses, the hepatitis lesions and liver necrosis were observed in experimental mice.

Discussion: The thiazole derivatives possessed more inhibitory trace on gram positive bacteria than gram negative ones. Furthermore, the likely presence of oxygen link to thiazole ring in tested compounds results in the enhancement of inhibitory potency of these substances. Besides, our results suggest that high doses of oxothiazole cause severe liver damage and rapid death less than 24 hours.

Keywords: Antibacterial Effects, Oxothiazole, Liver Toxicity.

INTRODUCTION

Staphylococcus aureus are gram positive bacteria residing on skin and in the upper respiratory tracts which several million of nosocomial infections are appointed to this organism (1). Also these bacteria are of great importance to humans due to the enterotoxin production in food products and its related poisoning condition (2). Although the antibiotic resistance of *Staphylococcus aureus* to beta-lactams, aminoglycosides and macrolides has resulted in the increased mortality and health charges in many nations (3). Another gram positive bacteria are *Streptococcus agalactiae* which cause meningitis and septicemia in fetus (4). *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are two gram negative bacteria which are involved as pathogen in nosocomial and respiratory secondary infections, respectively (5, 6). The antibiotic resistance in all of these pathogens has been observed in many countries globally (5-7). The health threat on the one hand and high costs of treatment on the other hand, which are often seen in nosocomial infections, have attracted the attention of scientists to novel antibacterial compounds in order to harness the resistant bacteria. Thiazole derivatives are among these unprecedented substances. Thiazole is a circular compound with a wide range of derivatives, possessing some biological roles such as the presence in vitamin B₁, anti-tumor signature, anti-HIV and effects opposing the hypertension (8, 9). However recent studies have indicated to the anti-inflammatory and antioxidant properties of these compounds (10, 11) and their potency has been demonstrated in-vitro to inhibit fungal pathogens like *Candidia* and *Aspergillus* as well as parasitic pathogens such as *Trypanosoma* and *Anopheles* mosquito (12, 13, 14). Antibacterial effects of thiazole compounds have recently been considered and many of their derivatives have been examined on various gram positive and gram negative bacteria such as *Staphylococcus aureus*, *staphylococcus pyogenes*, *Escherichia coli* and *Enterococcus*

pyogenes in order to distinguish the antibacterial characteristics (15, 16). Unfortunately most of the studies on antibacterial properties of thiazole derivatives have been confined to laboratory and lesser clinical trials on laboratory animals have caused little information be available on the toxic and therapeutic effects of these compounds. Despite of the potent antibacterial properties of thiazole derivatives, the aforementioned issue has resulted in their slender functional application in treating the bacterial diseases. At first, the goal of this study was to evaluate the inhibitory effect of new thiazole substance named oxothiazole (derivative 6d) on *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* by measuring the diameter of growth inhibition zone and MIC. In order to better comparison of oxothiazole antibacterial characteristics, the antibacterial effect of the other thiazole compounds (derivatives 6a-c which all of their construction phases are similar to those of oxothiazole except of the last stage; derivatives 9 and 10a-c that are equivalent to oxothiazole structurally in the case of the presence of thiazole ring) was also unraveled along with the oxothiazole antibacterial clue (figure 1). Then to better recognize the oxothiazole toxicity effects in high injection doses, liver as the most important organ confronting many toxins and drugs was histopathologically assessed after intra peritoneal (IP) injection in mice to declare the toxicity of this novel thiazole compound alongside discerning its antibacterial effect.

MATERIAL AND METHODS

Thiazole derivatives were synthesized in the following way:

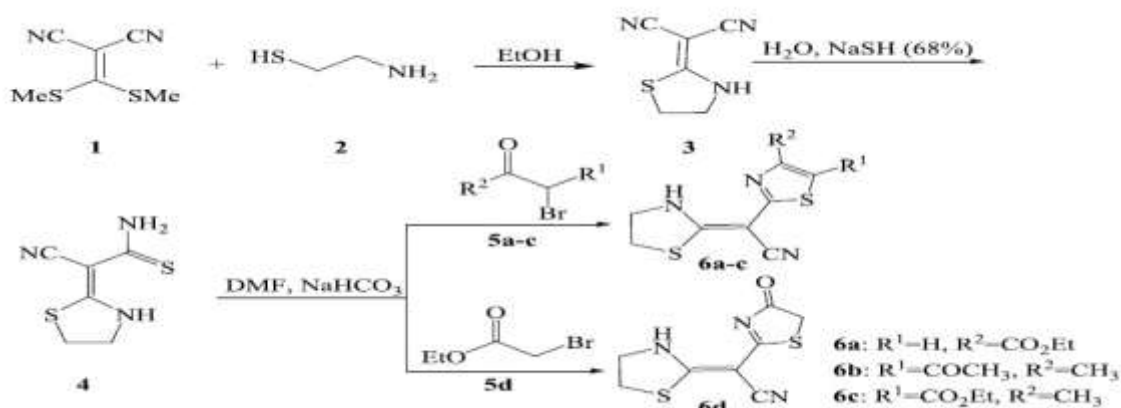
Synthesis of 2-(E)-cyano(thiazolidin-2-ylidene) thiazoles (6a-d)

2-(E)-Cyano(thiazolidin-2-ylidene)thiazoles¹ (6a-d) were prepared in a three-step procedure starting from the dinitrile (fig. 1. No, 1). (Figure 1). Cyclocondensation of 2-[bis(methylthio)methylene]malononitrile (fig. 1. No, 1) and cysteamine (fig. 1. No, 2) in ethanol

afforded thiazolidine (fig. 1. No. 3), which was filtered off and thionated by sodium hydrosulfide hydrate in water to give (*E*)-2-cyano-2-(thiazolidin-2-ylidene)ethanethioamide (fig. 1. No. 4) in a regioselective manner on the basis of its single crystal X-ray diffraction analysis, reaction of this compound with α -

bromocarbonyl compounds 5a-d in DMF as a solvent gave 6a-d. Structures are confirmed for these compounds on the basis of spectral analysis such as: single crystal X-ray diffraction, ^1H NMR, ^{13}C NMR, IR, elemental analysis, mass spectroscopy. Solutions were prepared by dissolving derivatives in DMSO as a solvent (8).

Figure 1- Synthesis of 2-(*E*)-cyano(thiazolidin-2-ylidene)thiazoles (6a-d)



6a: ethyl 2-[(*E*)-ciano(thiazolidine-2-ilidine)methyl]thiazole-4-carboxylate

6b: (*E*)-2-(5-acetyl-4-methyl thiazole-2-il)-2-thiazolidine-2-ilidine)acetonitril

6c: ethyl-2-(*E*)-ciano(thiazolidine-2-ilidine)methyl)-4-methyl thiazole-5 carboxylate

6d: (2*E*)-2-(4,5dihydro-4-oxothiazole-2-il)-2-(thiazolidine-2-ilidine)acetonitril

figure 2-Synthesis of 2-[(*E*)-(benzo[d]thiazol-2(3*H*)-ylidene)(cyano)methyl]thiazoles (10a-c)



9: (*E*)-2-(benzo[d]thiazole-2(3*H*)-ilidine)-2-cyano ethan thioamide

10a: ethyl 2-[(*E*)-benzo-[d]thiazole-2(3*H*)-ilidine)(cyano)methyl]thiazole-2-carboxylate

10b: (*E*)-2-(5-acetyl-4 methyl thiazole-2-il)-2-(benzo[d]thiazole-2(3*H*)-ilidine)acetonitrile

10c: ethyl 2-[(*E*)-benzo[d]thiazole-2(3*H*)-ilidine)(cyano)methyl]-4-methyl thiazole-5-carboxylate

Table 1- Diameter of growth inhibition zone (mm) of thiazole derivatives and antibiotics on *Staphylococcus aureus* and *Streptococcus agalactiae*.

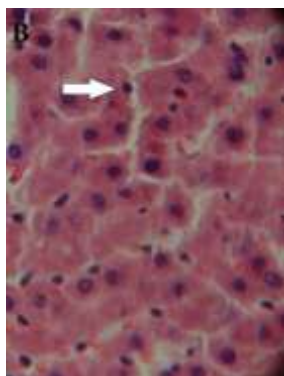
| Thiazole derivatives and antibiotics | <i>Staphylococcus aureus</i> (1189) | <i>Streptococcus agalactiae</i> (1768) |
|--------------------------------------|--|---|
| 6a-c | - | - |
| 6d | 22.3 ± 0.1 | 16.1 ± 0.2 |
| 9 | 18.1 ± 0.0 | 25.6 ± 0.3 |
| 10a | 8.9 ± 0.2 | - |
| 10b | 10.1 ± 0.4 | - |
| 10c | 12.8 ± 0.3 | - |
| Neomycin (30 µg) | 13.3 ± 0.0 | 17.5 ± 0.1 |
| Thylozine (30 µg) | 24.2 ± 0.4 | 29.4 ± 0.2 |
| Tetracycline (30 µg) | 34.1 ± 0.0 | - |
| Gentamycin (10 µg) | 14.8 ± 0.4 | 18.3 ± 0.2 |
| Lincomycin (2 µg) | 29.7 ± 0.0 | - |
| Florfenicol (30 µg) | 30.1 ± 0.1 | 40.6 ± 0.2 |
| Penicillin (10 µg) | 31.4 ± 0.3 | 29.6 ± 0.1 |
| Ampicillin (10 µg) | 33.2 ± 0.3 | 31.3 ± 0.2 |

Table 2- MIC (µg/ml) of thiazole derivatives on *Staphylococcus aureus* and *Streptococcus agalactiae*.

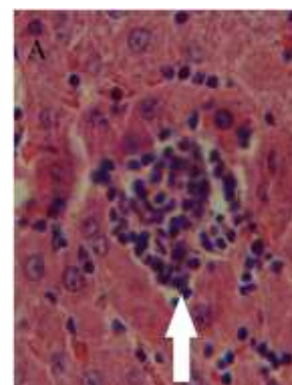
| Thiazole derivatives | <i>Staphylococcus aureus</i> (1189) | <i>Streptococcus agalactiae</i> (1768) |
|----------------------|--|---|
| 6a-c | - | - |
| 6d | 50 | 100 |
| 9 | 100 | 25 |
| 10a | - | - |
| 10b | 200 | - |
| 10c | - | - |

(-) : Due to not observing inhibitory effect in well diffusion method, MIC was not calculated.

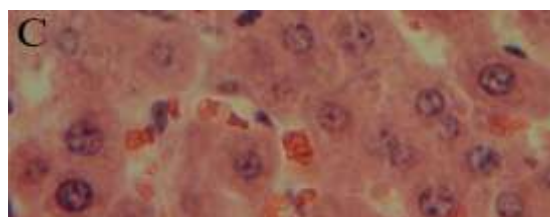
Histopathology images of examined mice liver



Hepatocyte necrosis in mice after injection oxothiazole thiazolidine



Hepatitis in mice liver after injection oxothiazole thiazolidine



Normal liver in mice of control group after DMSO injection

Synthesis of 2-[(E)-(benzo[d]thiazol-2(3H)-ylidene)(cyano)methyl]thiazoles (10a-c) 2-[(E)-(Benzo[d]thiazol-2(3H)-ylidene)(cyano)methyl]thiazoles (10a-c) were prepared in a three-step procedure starting from the dinitrile (fig. 2. N, 1), (Scheme, 2). Reaction of 2-[bis(methylthio)methylene]malononitrile (fig. 2. No, 1), and 2-aminothiophenol (fig. 2. No, 7), in ethanol afforded benzo[d]thiazole (fig. 2. No, 8), which was filtered off and thionated by phosphorus pentasulfide in ethanole to give (E)-2-(benzo[d]thiazol-2(3H)-ylidene)-2-cyanoethanethioamide (fig. 2. No, 9), in a regioselective manner on the basis of our previously reported work, reaction of this compound with various α -bromocarbonyl compounds 5a-c in DMF as a solvent gave 10a-c. Structures are confirmed for these compounds on the basis of spectral analysis such as: ^1H NMR, IR, elemental analysis, mass spectroscopy. Solutions were prepared by dissolving derivatives in DMSO as a solvent (9).

Preparation of the bacterial suspension

The examined bacteria, *Staphylococcus aureus* (PTCC 1189), *Streptococcus agalactiae* (PTCC 1768), *Pseudomonas aeruginosa* (PTCC 1310) and *Klebsiella pneumoniae* (PTCC 1290), were obtained from Persian Type Culture Collection (PTCC), Tehran, Iran.. Thereafter, each organism was cultivated in Mueller Hinton agar medium in 37 °C for 24 hours. Then, in Mueller Hinton broth medium under sterile condition a concentration of 0.5 McFarland (1.5×10^8 CFU/ml) was obtained with spectrophotometer and standard McFarland tube number 0.5 from each organism which was assigned to as a stock solution (17).

Discerning the minimum inhibitory concentration (MIC)

The MIC experiment was performed in a sterile 96-well plate by means of broth microdilution method according to CLSI standard. First, 100 μl of Mueller Hinton broth medium (Merck® - Germany) was added to each well. Afterwards, 100 μl of thiazole derivatives were incorporated to the first well and after mixing, 100 μl of this admixture was embedded to the second well.

This dilution procedure was fulfilled in other wells, similarly. Finally, 10 μl of bacterial suspension was added to each well. The results of incubation were read after 24 hours incubation in 37 °C. The lucidity and turbidity in each well suggested lack or existence of bacterial growth, respectively. The last well that didn't show any turbidity, was reported as MIC.

Assigning the diameter of growth inhibition zone

At first, the superficial bacterial culture was exerted in Mueller Hinton agar medium by a swab applied to bacterial suspension. Then 20 μl of thiazole derivatives solution were shed on sterile blank disks by means of a sampler. Padtan Teb company antibiotic disks were used for comparison and the diameter of growth inhibition zone was calculated by a particular ruler after 24h incubation in 37 °C. The desired results have been represented as mean \pm standard deviation which data were analyzed by SPSS statistical software ver. 22 (17).

In-vivo study

For the aim of in-vivo study, 24 white male BALB/c mice weighting $30 \pm 4\text{g}$ were obtained randomly from the animal house of veterinary college. Mice were maintained with access to standard food and refined water until the final stage of experiment in 25 ± 2 °C temperature, 50% humidity, 12 hours brightness and 12 hours darkness (18).

After initial test with different oxothiazole doses by up and down method, finally three doses of 350 mg/kg (death during 24h), 265 mg/kg (death during 72h) and 160 mg/kg (death is not observed during 72h) were selected for intra peritoneal injection. Animals were assorted to 4 groups each containing 6 mice and group A received 160 mg/kg, group B received 265 mg/kg, group C were conferred 350 mg/kg of oxothiazole (6d) and group D were entitled as control, receiving only DMSO solvent (18).

Histopathologic examination

After 24-48h, mice were anesthetized by ether and the abdominal wall was excised by scissors. The liver was taken out from the abdominal cavity and was placed in formalin 10%. Every 24h the maintaining formalin

solution was replaced till 72h. According to histopathologic principles the histological sections were prepared and after molding with paraffin, sections were processed with a diameter of 4-6 μm . The prepared slides were stained and examined with Hematoxylin and Eosin (H & E) under a light microscope (19).

RESULTS

None of the derivatives had a significant inhibitory effect on *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Compounds 6a-c also were not able to exert inhibitory clue on none of the examined bacteria. Additionally, substances 10a-c were effective only on *Staphylococcus aureus*. Derivative 6d with MIC 50 $\mu\text{g/ml}$ and derivative 9 with MIC 25 $\mu\text{g/ml}$ possessed the most inhibitory action on *Staphylococcus aureus* and *Streptococcus agalactiae*, respectively. Comply with antibiogram test, the most and the least inhibitory function on *Staphylococcus aureus* organism were related to tetracycline and gentamycin, respectively. Furthermore, florfenicol and neomycin own respectively the most and the least inhibitory effect on *Streptococcus agalactiae*. The results of the halo diameter for derivatives and antibiotics have been represented in Table 1 and the results related to MIC calculations have been shown in Table 2. In liver histopathologic examination of tested mice it was observed that increasing the injection dose of oxothiazole from 160 mg/kg to 350 mg/kg elicits infiltration of inflammatory cells to liver and consequently provokes the incidence of hepatitis (figure A) and necrosis of liver parenchyma (figure B). The mortality rate of 160, 265 and 350 mg/kg doses were none, 4 and 6 cases during 72h. There wasn't observed any lesion or mortality in control group and the context of liver was normal (figure C).

DISCUSSION

Thiazole derivatives possess various characteristics which their most known properties is the antibacterial effect. In this regard synthesizing and performing microbiological tests and evaluating the toxicity of compounds have been popular in many nations. In this research the antimicrobial and

toxicity tests were accomplished using newly synthesized thiazole derivatives in chemistry laboratory and according to standard methods. Our results suggest that the inhibitory action of compounds is confined to examined gram positive bacteria and it's not been applied any inhibitory function on gram negative bacteria, whereas many other thiazole derivatives examined for their antibacterial effect in recent years have impacts on both gram positive and gram negative bacteria. For instance, Liaras *et al.* indicated the inhibitory potency of thiazole derivatives on a wide range of gram positive bacteria like *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus subtilis* as well as gram negative organisms such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium* by calculating MIC (20).

The most potent inhibitory effect on *Staphylococcus aureus* was related to derivative 6d and its structure analysis shows that beside the presence of thiazole ring, two other important structures also exist. First, the presence of thiazolidine ring, similar to that in the structure of beta-lactams antibiotic family and the thiazolidine derivatives are recognized as having a wide range effect on gram positive bacteria like staphylococci and streptococci and recent studies have clarified the inhibitory action of these derivatives on *Staphylococcus aureus* and *Streptococcus agalactiae* (21). Second, the presence of oxygen link to thiazole ring which have established the oxothiazole which is exclusively incorporated in the structure of derivative 6d among compounds 6a-d and the inhibitory potency of oxothiazole-embedded derivatives have been substantiated on *Escherichia coli*, whereas the oxothiazole derivative (compound 6d) lack any inhibitory effect on gram negative bacteria (22). Derivative 9 possessed the most inhibitory function on *Streptococcus agalactiae* and the structure difference of this thiazole derivative with compounds 10a-c which couldn't influence *Streptococcus agalactiae*, was the linkage of thioamide branch to thiazole ring suggesting the outstanding active biological role of thioamide in some drugs such as anti-mycobacterium

perthioamide (23). Aggrawal *et al.* determined the diameter of growth inhibition zone to assess the antibacterial effect of thiazole derivatives which their results indicated to the compounds inhibitory action with halo diameter of 1.87 – 4.5 mm on *Staphylococcus aureus*, 7.87 – 9.13 mm on *Pseudomonas aeruginosa* and 10.10 – 13 mm on *Klebsiella aerogenes* but the blocking potency of these derivatives on gram positive *Staphylococcus aureus* is lesser than our derivatives. However the thiazole derivatives of Aggrawal *et al.* experiment possessed some blocking characteristics on gram negative bacteria which probably the presence pyrazole ring along with thiazole ring in this compound has been effective to influence bacteria (24). Abbasi shiran *et al.* mentioned the effectiveness of thiazole compounds on both gram negative and gram positive bacteria by representing the antibacterial effect of thiazole derivatives *Salmonella enterica*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* by calculating MIC (25). The blocking potency of thiazole derivatives was proved on *Bacillus thuringiensis* and *Escherichia coli* by Khalil *et al.* with measuring the diameter of growth inhibition zone which in this study the effect of one compound was conspicuous in comparison to derivatives 6d and 9 in our research in which diphenil, phetalazine and oxygen junctions probably enhance the thiazole antibacterial properties in this derivative (26). The gathered results by our work in which the derivatives could only inhibit gram positive bacteria, strengthens this hypothesis that these compounds overwhelm and inhibit the cell wall regarding to their antibacterial effects but researches on thiazole antibacterial impacts have shown that these compounds exert their functions by enzyme inhibition such as DNA Gyrase B enzyme (The quinolone antibiotics block DNA Gyrase A and we can possibly get help from thiazole derivatives either against quinolone-resistant bacteria or enhancing their synergistic effect alongside quinolone antibiotics) or impeding expression of specific genes such as *fabH* (possessing a crucial role in fatty acid metabolism in bacteria) (27, 28). In

contrast with oxothiazole which causes rigorous damage to liver, Chen *et al.* showed that one of the thiazolidine derivatives named SKLB010 owing to its strong antioxidant and anti-inflammatory properties plays an important role to protect liver against carbon tetrachloride (CCl₄) and prevents from liver necrosis and hepatitis due to the CCl₄ (29). Assessing 12 used thiazolidine derivatives, only 3 derivatives could protect liver because of their potent anti-inflammatory characteristics and the criterion for anti-inflammatory effect in this study was measuring released nitric oxide (NO) from macrophages that likely oxothiazole thiazolidine not only lack anti-inflammatory properties but also provoke inflammation by damaging liver (30). Using thiazole-Zn (a systemic fungicide) as oral gavage in female rats lack any pathologic impressions in liver and the only toxicity effect was observed in thyroid gland (19).

CONCLUSION

Regarding to lack of inhibitory action of thiazole derivatives on gram negative bacteria in this study, evaluating the effects of these compounds and performing other complementary experiments in order to discriminate the mechanism of their antibacterial function is a requisite. Furthermore, wide range characteristics of thiazole derivatives on body organs and functions such as blocking ionic channels, enzyme inhibition and cellular organelles (31-34) beside higher dose of oxothiazole used in mice could be accounted for liver damage incidence in the time of injection of this compound and discerning toxic and therapeutic effects of oxothiazole seeks much more in-vitro and then in-vivo experiments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest between them.

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