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# Novel Synthetic Derivatives of Dichloroimidazole Targeting NorA Efflux Pump against Methicillin-Resistant *Staphylococcus aureus*

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## Abstract

**Introduction:**
Antibiotic resistance has been a major health problem in recent years, which has led to a failure in the treatment of infectious diseases. Therefore, research to synthesize compounds that have antibiotic activity is very valuable. In the present study, four novel compounds **(6a-d)**, derivatives of dichloroimidazole conjugated with triazole, were synthesized in order to obtain new bacterial efflux pump inhibitors (EPIs).

**Methods and Results:**
The derivatives were evaluated for their effects on the minimum inhibitory concentration (MIC) of ciprofloxacin against a methicillin and ciprofloxacin resistant *Staphylococcus aureus* (MCRSA) clinical isolate. Based on broth microdilution method assay, four derivatives at a minimum effective concentration (MEC) fortified the antibacterial efficacy of ciprofloxacin against MCRSA. MIC of ciprofloxacin decreased in the presence of novel compounds compared to ciprofloxacin alone between 2 to 64 fold. These compounds were then evaluated for their potency as efflux pump inhibitors using a fluorometric assay. Results indicated an increase in accumulation of ethidium bromide (a known fluorescent substrate for the NorA pump) in the presence of each compound, like verapamil (a typical inhibitor of efflux pump), thus these compounds acted as inhibitors of the NorA pump. Moreover, the MTT assay confirmed that novel compounds did not demonstrate any cytotoxic effect against three cancer cell lines, HT-29, MCF-7, Caco-2, and a normal mouse fibroblastic cell line, NIH-3T3.

**Conclusion:**
Collectively, our results propose these derivatives as therapeutic options in combination therapies to tackle antibiotic resistance.

**Keywords:** Synthesis, Triazoles, Antibacterial activity, Cytotoxic activity, Inhibitors

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## 1. Introduction

During the past decades, resistance to antimicrobial agents has augmented remarkably throughout the world, causing major problems in chemotherapy of infectious diseases [1]. One of the most important resistances has been reported in methicillin-resistant *Staphylococcus aureus* (MRSA) that plays a crucial role in both community and hospital-acquired infections [2, 3]. Thus, extensive studies were conducted to overcome the obstacles related to the outbreak of antibiotic resistance in MRSA [1].

Antibiotic resistance in *S. aureus* has been mediated through various mechanisms, among which bacterial efflux pumps are considered to be important factors in resistance to different classes of antibiotics and other antimicrobial compounds. These pumps are

transmembrane proteins that detoxify cells mediated by extruding of antibiotics and decrease their intracellular concentration [4, 5] which may produce multidrug resistance (MDR) in bacteria [3, 6]. Several cases of these pumps have been reported in *S. aureus*, causing drug resistance such as NorA, NorB, NorC, and MdeA that belongs to the Major Facilitator Superfamily (MFS) as well as MepA from the multidrug and toxic compound extrusion (MATE) family [7]. Accordingly, *S. aureus* is less sensitive to fluoroquinolone antibiotics (e.g., ciprofloxacin) due to the active exclusion from the cell by the NorA MDR efflux pump [4, 8].

The NorA efflux pump of *S. aureus* causes resistance to a broad series of dissimilar substrates [9, 10] including hydrophilic fluoroquinolones (ciprofloxacin, norfloxacin) [11], biocides (acriflavine, cetrimide, benzalkonium chloride) [10, 12], verapamil [13], reserpine, and dyes (ethidium bromide, rhodamine, acridines) [14].

Consequently, particular attention has been focused to surmount resistance mechanisms such as the use of the efflux pump inhibitors (EPIs) [15, 16]. Use of these compounds in combination therapy helps to enhance the efficacy of antibiotics and decrease their therapeutic doses [14, 17]. In this regard, numerous researches have started deriving various EPIs from natural products (such as phytochemicals) and produce synthetic compounds [18, 19]. It has been observed that some of these compounds have the ability to increase the effectiveness of antimicrobial agents in combination therapies [1, 20] and thereby reduce the effective therapeutic dose of antimicrobial drugs [17, 21]. Recent studies have reported the dicholoroimidazole and 1,2,4-triazole derivatives with antimicrobial [22, 23] and MDR inhibitory effects [24, 25]. In the present study, we synthesized four novel derivatives of dichloroimidazole conjugated with triazole and evaluated their effects on the minimum inhibitory concentration (MIC) of ciprofloxacin. Following *in-vitro* cytotoxicity study, efflux assay was performed to assess the aforementioned compounds as novel EPIs.

## 2. Materials and Methods

### *2.1. General remarks*

Melting points were determined with a Reichert-hot stage microscope and are uncorrected. $^1$H NMR spectra were recorded on a 500 MHz Brucker spectrometer using $CDCl_3$ or DMSO-$d_6$ as a solvent. Chemical shifts (δ) are reported in ppm relative to TMS as an internal standard. Infra spectra were acquired on a Nicolet Magna 550-FT-spectrometer. Mass spectra were obtained with a Finnigan Mat TSQ-70 spectrometer. Elemental analysis was within ± 0.4% of the theoretical values for C, H, and N. All other chemicals for synthesis were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO; Merck) was used to prepare the stock solutions of derivatives. The final concentration of DMSO was less than 1-2% without any undesirable effect on cell viability. Ciprofloxacin and verapamil were purchased from Shahid Ghazi and Rouzdaro Co. (Tehran, Iran), respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO, USA).

### *2.2. Synthesis*

#### *2.2.1. (4,5-dichloroimidazole-1-yl) propionic acid methyl ester **(3)***

To a solution of compound **1** [26], (0.1 mol, 13.7 g) in 200 mL chloroform, 0.1 mol of methyl acrylate (8.6 g), and 5 mL triethylamine were added and refluxed for 16 h.

The solvent was removed under reduced-pressure. Purification was carried out by column chromatography on silica using a mixture of dichloromethane and hexane as mobile phase. The mixture was crystallized from ether :n-hexane (3:1 v/v) to give 20.4 g (92%) of compound **3**.

m. p. = 45-47 °C, $^1$H NMR ($CDCl_3$) δ: 2.8 (t, J = 7.5 Hz, 2H, $CH_2$-CO), 3.70 (s, 3H, $OCH_3$), 4.25 (t, J = 7.5 Hz, 2H), 7.47 (s, 1H, H-imidazole). IR (KBr) V: 1738 $cm^{-1}$. Mass: m/z (%) 222 (61), 191 (20), 149 (38), 113 (100), 86 (60).

### *2.2.2. (4,5-dichloroimidazole-1-yl) propionic acid hydrazide **(4)***

A solution of **3** (0.03 mol) in methanol (20 mL) was stirred in a cold water bath and then, 1.5 mL of hydrazine 98% was added. The mixture was stirred for 3 h, and the precipitate was collected and washed with a few mL of methanol and chloroform. The yield was 80%. m. p. = 65-67 °C $^1$H NMR (DMSO-$d_6$) δ: 2.51 (t, J = 7.0 Hz, 2H), 4.19 (t, J = 7.0 Hz, 2H), 7.70 (s, 1H, H-imidazole), 9.11 (bs, 1H, NH). IR (KBr) °C: 3328, 3180, 3099, 1678 cm$^{-1}$. Mass: m/z (%) 222 (30), 187 (45), 163 (75), 150 (100), 85 (43), 67 (72).

### *2.2.3. General Procedure for N1-aryl-N2-(4,5-dichloroimidazole-1-yl) propionic acid) carboxamide thiourea **(5a-d)***

To a solution of **4** (0.01 mol) in 2 mL of ethanol, aryl isothiocyanates (0.01 mol), and 5 mL of NaOH solution (2N) were added. The mixture was stirred for 3 h at room temperature. The pH was adjusted to 2 by adding concentrated hydrogen chloride solution. The mixture was stored at 4 °C for 24 h. Related thiosemicarbazides were removed by filtration and directly used for next step.

### *2.2.4. General procedures For 4-aryl-5-(4,5-dichloroimidazole-1-yl-ethyl)-2,4-dihydro-[1,2,4]-triazole-3-thiones **(6a-d)***

To 0.01 moles of compound **5** 10 mL of saturated sodium carbonate solution was added and refluxed for 5 h. After cooling, hydrogen chloride was added and, the precipitate was filtered. Crystallization from methanol and water gave compounds **6**.

#### *2.2.4.1. 4-(Phenyl)-5-(4,5-dichloroimidazole-1-yl-ethyl)-2,4-dihydro-[1,2,4]-triazole-3-thione **(6a)***

% yield = 78, m. p. = 240-241 °C, $^1$H NMR (DMSO-$d_6$) δ: 2.70 (t, J = 6.8 Hz, 2H, $CH_2$), 4.23 (t, J = 6.8 Hz, 2H, $CH_2$), 7.16 (m, 3H), 7.36 (m, 2H), 7.75 (s, 1H, H-imidazole), 9.5 (bs, 1H, NH), IR (KBr) V: 3656, 3250, 1690 cm$^{-1}$, Mass: m/z (%) 264 (10), 227 (18), 186 (25), 149 (20), 135 (100), 98 (20), 77 (55), Anal. Calcd. for $C_{13}H_{11}Cl_2N_5S$: C, 45.89; H, 3.25; N, 20.58. Found: C, 45.65; H, 3.21, N, 20.50.

#### *2.2.4.2. 4-(4-Nitrophenyl)-5-(4,5-dichloroimidazole-1-yl-ethyl)-2,4-dihydro-[1,2,4]-triazole-3-thione **(6b)***

% yield = 90, m. p. = 120-123 °C, $^1$H NMR (DMSO-$d_6$) δ: 2.73 (t, J = 6.4 Hz , 3H, $CH_2$), 4.25 (t, J = 6.4 Hz , 2H, $CH_2$), 7.75 (s, 1H, H-imidazole), 7.86 (d, J = 7.8 Hz , 2H), 8.20 (d, J = 7.8 Hz , 2H), 10.05 (bs, 1H, NH). IR (KBr) V: 3322, 1664, 1530, 1348 cm$^{-1}$, Mass: m/z (%) 265 (5), 222 (10), 181 (65), 135 (100), 91 (79), 66 (85), Anal. Calcd. for $C_{13}H_{10}Cl_2N_6O_2S$: C, 40.53; H, 2.62; N, 21.82. Found: C, 40.66; H, 2.47, N, 21.75.

#### *2.2.4.3. 4-(4-Chlorophenyl)-5-(4,5-dichloroimidazole-1-yl-ethyl)-2,4-dihydro-[1,2,4]-triazole-3-thione **(6c)***

% yield = 70, m. p. = 175-177 °C, $^1$H NMR (DMSO-$d_6$) δ: 2.73 (t, J = 7.1 Hz, 2H, $CH_2$), 4.30 (t, J = 7.1 Hz, 2H, $CH_2$), 7. 5 (m, 4H), 7.78 (s, 1H, H-imidazole), 9.6 (bs, 1H, NH), IR (KBr) V: 3367, 1680 cm$^{-1}$, Mass: m/z (%) 264 (10), 246 (15), 225 (10), 187 (15), 169 (100), 112 (38), 76 (39), Anal. Calcd. for $C_{13}H_{10}Cl_3N_5S$: C, 41.67; H, 2.69; N, 18.69. Found: C, 41.59; H, 2.50, N, 18.75.

#### *2.2.4.4.4-(4-Methoxyphenyl)-5-(4,5-dichloroimidazole-1-yl-ethyl)-2,4-dihydro-[1,2,4]-triazole-3-thione **(6d)***

% yield = 90 m. p. = 161-163 °C, $^1$H NMR (DMSO-$d_6$) δ: 2.96 (t, J = 7.1 Hz , 2H, $CH_2$), 3.75 (s, 3H, $OCH_3$), 4.22 (t, J = 7.0 Hz , 2H, $CH_2$), 6.90 (d, J = 7.5 Hz , 2H), 7.22 (d, J = 7.5 Hz , 2H), 7.75 (s, 1H, H-imidazole), 9.6 (bs, 1H, NH), IR (KBr) V: 3322, 16646, 1530, 1348 cm$^{-1}$, Mass: m/z (%) 370 (M+, 2), 222 (5), 186 (20), 186 (20), 165 (95), 151 (58), 124 (100), Anal. Calcd. for $C_{14}H_{13}Cl_2N_5OS$: C, 45.41; H, 3.54; N, 18.92. Found: C, 44.35, H, 3.54, N, 18.92.

### *2.3. Biology*

#### *2.3.1. Bacterial strain and media*

Ciprofloxacin and methicillin-resistant clinical isolate of *S. aureus* was obtained from the Imam Khomeini

Hospital, Karaj. The resistance of MRSA strain was confirmed by the Kirby-Bauer disk-diffusion assay. The following antibiotic disks were consumed: methicillin (30 µg/ mL), ciprofloxacin (5 µg/ mL), and oxacillin (1 µg/ mL) (Pad Tan Teb, Tehran, Iran). For determination of MICs and ethidium bromide (EtBr) accumulation experiments, the cultures were grown in Muller-Hinton broth (MHB; Merck) at 37 °C.

### *2.3.2. Cell culture*

The human colon adenocarcinoma (HT-29), human breast adenocarcinoma (MCF-7), colorectal adenocarcinoma (Caco-2), and a mouse embryonic fibroblast cell line (NIH-3T3) [27, 28] were obtained from National Cell Bank, Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI-1640 medium (Biosera, East Sussex, UK), containing 10% heat-inactivated Fetal Bovine Serum (FBS; Gibco, CA, USA), 1% penicillin-streptomycin (Biosera) and were kept in humidified 5% $CO_2$ incubator at 37 °C. However, Caco-2 cells were maintained in 50% RPMI-1640, 35% DMEM/F12 (Biosera), 15% FBS, and 1% penicillin-streptomycin.

### *2.3.3. Determination of MICs*

In this study, MICs of ciprofloxacin and derivatives against MCRSA were determined by the broth micro-dilution method based on the criteria of Clinical and Laboratory Standards Institute (CLSI) [3]. Briefly, suspension equal to a 0.5 McFarland standards was prepared ($10^6$ CFU/ mL *S. aureus* were inoculated into MHB) from the culture which was incubated for 18 h at 37 °C. Then, the suspension was diluted to give the final concentration of $5 \times 10^5$ CFU/ mL and was added to a serial dilution of the ciprofloxacin (0.25-500 µg/ mL) and **6a-d** (1.22-250 µg/ mL) in 96-well plates. MIC value was determined as the lowest concentration with no visible growth (no obvious turbidity). Afterwards, the effect of compounds **6a-d** on the MIC of ciprofloxacin was evaluated. For this purpose, MIC of ciprofloxacin in combination with 1/2 MIC of **6a-d** was specified by the broth micro-dilution method against MCRSA.

### *2.3.4. Effect of compounds on accumulation of EtBr*

In order to assess the effect of compounds as efflux pump inhibitors, EtBr accumulation measurement was performed by fluorimetry. Since verapamil is a known efflux inhibitor, it was used as a positive control at a concentration of 100 µg/ mL [2, 29]. Therefore, the level of EtBr accumulation in MCRSA in the presence and absence of inhibitors was evaluated according to previous reports [4, 15, 30]. Briefly, bacterial suspensions with an optical density of 0.2 at 550 nm were ready in uptake buffer (110 mM NaCl, 7 mM KCl, 50 mM $NH_4Cl$, 0.4 mM $Na_2HPO_4$, 52 mM Tris base and 0.2% glucose, adjusted to pH 7.5 with HCl). Then, the suspensions were exposed to EtBr (Merck) at a concentration of 2 µg/ mL with EPIs at MEC (minimal concentration of EPIs that produced the maximal reduction in MIC of ciprofloxacin, for compound **6a** and **6b** at 78.125 µg/ mL, for compound **6c** at 19.53 µg/ mL and for compound **6d** at 156.25 µg/ mL). Upon EtBr penetration into the cells, the increase in fluorescence was measured spectrophotometrically for fourty five minutes at intervals of five minutes while 530 nm and 600 nm were adjusted for excitation and emission wavelengths, respectively.

### *2.3.5. Cell viability assay*

Since the antibacterial agents should demonstrate no or confined harmful side effects against the host cell, the effects of new derivatives on four different cell lines (HT-29, MCF-7, Caco-2, and NIH-3T3) were evaluated by conventional MTT assay [8, 28]. The cells (8000-1000 cells/well) were seeded into 96-well plates and incubated at 37 °C for 48 h. Subsequently, the cells were treated with different concentrations of each compound (62.5-1000 µg/ mL). Following 48 h incubation, the medium was discarded and 20 µL of MTT solution (5 mg/ mL in PBS) was added to each well and incubated for additional 4 h. Then, 100 µL of DMSO was used to dissolve formazan crystals. Eventually, the absorbance was determined at 570 nm with a reference wavelength of 690 nm by a plate reader (Anthos 2020, Biochrom, Cambridge, UK). The cytotoxicity is expressed as the concentration of compounds which inhibits the growth of 50% of cells

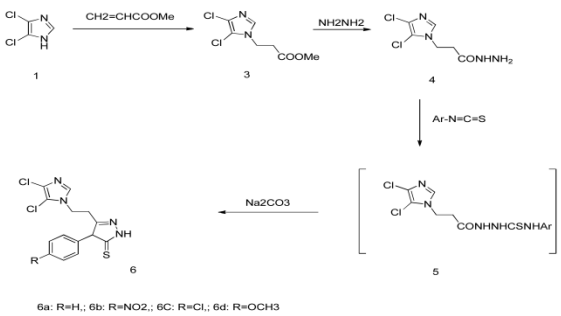
compared with vehicle-treated cells. The results were presented as the mean ± standard deviations for at least three independent experiments performed at least in triplicate.



## 3. Results

### *3.1. Chemistry*

The previously reported reaction of aryl-hydrazide and the appropriate isothiocyanate was extended for the preparation of compounds **6** [31]. The synthetic reactions used for the preparation of 4-aryl-5-(4,5-dichloroimidazole-1-yl-ethyl)-2, 4-dihydro-1, 2, 4-triazole-3-thiones are outlined in scheme 1. Starting from (4,5-dichloroimidazole-1-yl) propionic acid hydrazide **4**, intermediate **5** were prepared. The compounds **5** were cyclized in saturated sodium carbonate solution to give the final compounds **(6a-d)** with good yield. Hydrized **4** was synthesized from compound **3** through the reaction with hydrazine. (4,5-Dichloroimidazole-1-yl) propionic acid methyl ester **3** was prepared from 4,5-dichloroimidazole **1** and methyl acrylate **2** through a Michel addition reaction.




**Scheme 1. Synthesis pathway for preparation of 1,2,4-triazole-3-thions**

### *3.2. Susceptibility study of MRSA strain and MIC determination*

Initially, the MIC value of ciprofloxacin against MCRSA was determined. We next defined the MIC of newly synthesized compounds to obtain concentration at which does not have any bacterial toxicity. Thus, their effectiveness was investigated only as EPIs. As summarized in Table 1, the MIC value of ciprofloxacin was 62.5 µg/mL while the MIC values of 156.5, 312.5, 1250, and 312.5 µg/ mL were determined for **6a-d** derivatives, respectively. Furthermore, MIC of ciprofloxacin (with a sub-inhibitory concentration) in combination with 1/2 MIC of compounds was evaluated using broth micro-dilution method against MCRSA. It was uncovered that MIC of ciprofloxacin decreased in the presence of novel compounds relative to ciprofloxacin alone with 2 to 64 fold decrease.

**Table 1. Minimum inhibitory concentration (MIC) values of ciprofloxacin alone and in combination with compounds 6a-d.**

| EPI | MIC of EPI (µg/ mL) | MEC of EPI (µg/ mL) | MIC of ciprofloxacin (µg/ mL) | | Reduction (*n*-fold) in MIC of ciprofloxacin |
|---|---|---|---|---|---|
| | | | Without EPI | With EPI | |
| 6a | 156.25 | 78.125 | 62.5 | 31.25 | 2 |
| 6b | 312.5 | 78.125 | 62.5 | 15.6 | 4 |
| 6c | 1250 | 19.53 | 62.5 | 0.97 | 64 |
| 6d | 312.5 | 156.25 | 62.5 | 31.25 | 2 |

### *3.3. Effect of EPIs on accumulation of EtBr*

Since the newly synthesized compounds decreased the MIC of ciprofloxacin, we examined their efficacy as inhibitors of NorA by evaluating their effects on accumulation of EtBr (a known fluorescent substrate for the NorA pump). The EtBr emits fluorescence upon binding to nucleic acids within the cells. An EPI compound leads to a significant increase due to the accumulation of EtBr in the bacteria [4, 32]. Figure 1 demonstrates the effect of the compounds on the accumulation of EtBr in comparison with the untreated control group as a negative control and verapamil as a positive control.

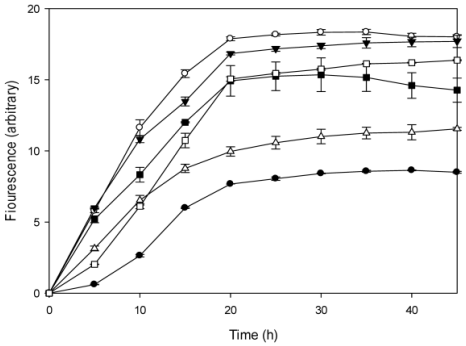


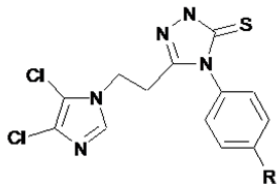

**Figure 1.** Accumulation of EtBr in MRSA in presence of compound **6a** (■), compound **6b** (□), compound **6c** (▼), compound **6d** (Δ), and verapamil (○) as a positive control, and without EPIs (●) as a negative control. Results were expressed as mean ± standard deviations for two independent experiments.

As shown in Figure 1, a remarkable increase in fluorescence was observed in the presence of each compound, though it was less than verapamil. This increase arises from the reduction of EtBr exit from the bacteria attributed to inhibition of efflux pump. While in control cells without EPIs, fluorescence was reduced due to efflux of EtBr.

### *3.4. Effect of compounds **6a-d** on in-vitro cytotoxicity*

The cytotoxicity was evaluated on four different cell lines (HT-29, MCF-7, Caco-2, and NIH-3T3) by treating the cells with different concentrations of the compounds **6a-d** using an MTT colorimetric assay (25). The results from three independent experiments were reported in Table 2. It was observed that most of the tested compounds at MEC concentration did not demonstrate cytotoxic activity against cancer cell lines. Only, exposure to increasing concentrations of compound **6b** abolished cell growth of MCF-7 with an $IC_{50}$ value of 115 ± 7.07 µg/ mL. In the case of normal cell line NIH-3T3, treatment with **6a-d** caused no or moderate cytotoxicity.

**Table 2. Structure and cytotoxicity of compounds 6a-d**




| | | Cytotoxicity ($IC_{50}$, µg/ mL)* | | | |
|---|---|---|---|---|---|
| Compounds | R | HT-29 | MCF-7 | Caco-2 | NIH-3T3 |
| 6a | H | 383.3 ± 4.9 | 239.5 ± 3.5 | 553 ± 31.8 | 338.5 ± 4.94 |
| 6b | $NO_2$ | >1000 | 115 ± 7.07 | >1000 | 95 ± 1.4 |
| 6c | Cl | 552 ± 2.82 | 213.33 ± 11 | 245 ± 7.07 | 264 ± 5.65 |
| 6d | $OCH_3$ | >1000 | >1000 | 636 ± 9.29 | 550 ± 30 |

* Values are the mean ± SD. All experiments were performed at least three times.

## 4. Discussion and Conclusion

One of the most important mechanisms of resistance elucidated in *S. aureus* is the efflux pumps, causing decrease in the intracellular concentration of antibiotics [2]. This phenomenon is considered as one of the main resistance factors to fluoroquinolones and other compounds in clinical isolates of *S. aureus* [15]. As a result, extensive researches have been conducted for synthesizing efflux pump inhibitors to prevent infections caused by *S. aureus* and emergence of new MDR strains [3]. In this regard, Chan et al. found that baicalein could significantly reduce the ciprofloxacin resistance in MRSA strains attributed to the NorA efflux pump inhibition [33]. Previous study clarified new analogues of piperine, an active component of *Piper nigrum*, as NorA pump inhibitors in *S. aureus* [4]. Moreover, Pourmand and colleagues demonstrated the hexahydroquinoline derivatives with the ability to decrease the MIC of ciprofloxacin and enhance the antibacterial effect of ciprofloxacin against MRSA [3].

There are some evidences that dicholoroimidazole and 1, 2, 4-triazole derivatives have demonstrated anti-microbial effects [22, 23]. Besides, MDR inhibition properties in triazole derivative were observed [24, 25]. In this study, we synthesized four novel derivatives of dichloroimidazole conjugated with triazole and investigated their antibacterial potency as EPIs. It should be noted that the sub-inhibitory concentration of ciprofloxacin and compounds **6a-d** was chosen to ensure that their effect was produced collectively not individually. Thus, the results obtained in these conditions could be due to the compounds intervention in antibiotic resistance. Our results indicated that these new compounds promoted the antibacterial activity of ciprofloxacin. Compounds **6a** and **d** caused a 2-fold decrease in MIC of ciprofloxacin whereas compound **6b** demonstrated a 4-fold decrease. Additionally, compound **6c** efficiently attenuated the MIC of ciprofloxacin with a 64-fold reduction. Although all compounds reduced the MIC of ciprofloxacin, this effect was noticeable for compound **6c**. Since these compounds are different only in one substitution, it seems that significant effect of compound **6c** arises from the existence of Cl substitution.

Regarding the previous studies corroborating the presence of efflux pump in *S. aureus* [12], compounds **6a-d** can alter the MIC of ciprofloxacin in MRSA by inhibition of this pump. On the other hand, it is recognized that MDR pumps of many Gram-positive bacteria such as NorA or NorB are transporter of EtBr. Thus, the effect of these new compounds on the efflux pump was evaluated by EtBr (resistance to EtBr is only mediated through the efflux pumps) [15, 34]. Our findings indicated that newly synthesized compounds led to the accumulation of EtBr compared with control cells without EPI in a similar way that verapamil does, implying their capability as the NorA suppressors. Similarly, Fontaine et al. investigated the effects of a series of boronic derivatives as efflux pump inhibitors against susceptible and resistant *S. aureus* strains. The results demonstrated that these compounds increased the activity of ciprofloxacin, norfloxacin, and reinforced EtBr accumulation [9]. In addition, it has been revealed that exposure to galbanic acid inhibits the efflux of EtBr in clinical isolates of *S. aureus* [15].

Since these candidates for use in combination therapy should not have cytotoxic effects, the cytotoxicity of them was evaluated against a panel of cell lines in the presence of compounds **6a-d**. Compounds **6a** and **d** inhibited less than 25% of the cell growth in four cell lines at MEC. Intriguingly, **6c** (the most promising compounds to inhibit the NorA efflux pump and reduce the MIC of ciprofloxacin) had the lowest level of cell growth inhibition and cytotoxicity (approximately less than 10% at MEC for all cell lines). Only compound **6b** demonstrated a moderate level of toxicity at MEC on MCF-7 and NIH-3T3 cell lines (35% and 45%, respectively).

In summary, four novel derivatives of dichloroimidazole were synthesized which have exerted significant potential for reducing the resistant of *S. aureus* strains and emerged as the efflux pump inhibitors. These new compounds particularly compound **6c**, the most effective compound with the lowest level of toxicity, can be used as putative inhibitors of antibiotic resistance in combination therapies against *S. aureus*. Considering the outbreak

of antibiotic resistance in MRSA strain, our findings have proposed compound **6c** as a plausible NorA inhibitor to overcome this obstacle. Moreover, further studies are needed to evaluate the expression of efflux pump gene in the presence of these compounds in MCRSA using molecular techniques.



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