Synthesis, Antibacterial Evaluation and Molecular Docking Study of Nitazoxanide Analogues

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A series of nitazoxanide analogues were synthesized and characterized by means of ^1H NMR, ^{13}C NMR and HR-MS. The antibacterial activities of synthesized compounds against *Clostridium difficile* were evaluated and **1d** was the most promising compound. The preliminary results showed that compounds containing nitro-substituted thiazole ring displayed notable activities. Compounds that thiazole moiety replaced by a pyrimidine ring exhibited moderate activities. Molecular docking study of nitazoxanide and **1d** with pyruvate ferredoxin oxidoreductase suggested that the nitro group interacts with thiamine pyrophosphate and surrounding amino acids, which were almost same compared with pyruvate. The results revealed that nitazoxanide may be a competitive inhibitor of pyruvate and nitro-group is necessary for thiazolidine's activities against anaerobic organisms containing pyruvate ferredoxin oxidoreductase enzyme.

Keywords: Nitazoxanide analogues, Synthesis, Antibacterial activity, Molecular docking.

INTRODUCTION

Parasitic and bacterial infections are the major cause of morbidity and mortality in the world especially in developing countries. Diseases caused by intestinal parasites affect billions of people every year and there have been few drug innovations for treating these infections in the past three decades. Fortunately, nitazoxanide (Fig. 1) belongs to nitrothiazole analogue was developed as a promising compound to treat these diseases. Nitazoxanide, 2-acetyloxy-N-(5-nitro-2-thiazolyl)benzamide was first synthesized by Rossignol and Cavier¹ and has shown activities against *Taenia saginata* and *Hymenolepis nana*². Further studies reported that nitazoxanide exhibited an unusually broad spectrum of activities against various parasites in human being and animals³⁻⁵. In 1996, nitazoxanide has been marketed in Latin America and then approved by the US food and drug administration as an agent treating infections caused by parasites^{6,7}. Nitazoxanide also showed broad anti-microbial action against anaerobic bacteria (*Clostridium difficile*, *Helicobacter pylori*) as well as notable activities against influenza virus and hepatitis virus⁸⁻¹¹. For highly pathogenic virus rotavirus and mycobacterium tuberculosis, studies have found that nitazoxanide can kill the virus or cure relative diseases^{12,13}.

In contrast to other nitro-drugs like metronidazole, nitazoxanide has been shown to be non-mutagenic, which suggested that the two class compounds have fundamental differences with the mode of action¹⁴. In fact, recent studies

Fig. 1. Nitro heterocyclic drugs

reveal that nitazoxanide inhibits pyruvate ferredoxin oxidoreductase, a key enzyme of central intermediary metabolism in anaerobic organisms. Nitazoxanide appears to interact with pyruvate ferredoxin oxidoreductase and the nitro group is not metabolically reduced. The mechanism was supposed that the amide anion (nitazoxanide ions) may directly couple with thiamine pyrophosphate, which is a cofactor of pyruvate ferredoxin oxidoreductase (Fig. 2)¹⁵. Recently, a number of nitazoxanide derivatives have been synthesized and their activities were also investigated¹⁶⁻¹⁷. The results demonstrated that the nitro group may be essential for the antimicrobial efficacy with a minimum inhibitory concentration of organisms

(MIC₉₀) of 0.06–4 mg/L¹⁸. Meanwhile, several analogues displayed more potent antiprotozoal and antiviral activities^{19–22}. But notably, specific replacements of the nitro group shown significant activities against intracellular parasites while invalidity for intestinal parasite *Giardia lamblia*²³. On the other hand, modifications of the salicylate moiety may reduce the activity even abrogate it²⁴. This indicated that other enzymes beside pyruvate ferredoxin oxidoreductase might also be relevant.

Fig. 2. Mechanism of pyruvate ferredoxin oxidoreductase enzymatic reaction

Owing to the structural specificity and notably efficacy, we hypothesized that modifications of the benzene ring and thiazole moiety may result increased bioactivities. In this work, the benzene ring hydrogen and nitro-group were substituted by halides, part of the compounds contained a pyrimidine ring instead of thiazole moiety. Actually, all the electron-withdrawing substituent can lead easily to the formation of nitazoxanide ions which can act on thiamine pyrophosphate causing the inhibition of pyruvate ferredoxin oxidoreductase. The antibacterial activities of these compounds are evaluated as a preliminary screening to provide some information about their antiparasitic efficiency, which is the final purpose of this project. In addition, a molecular docking study of nitazoxanide with pyruvate ferredoxin oxidoreductase was firstly established using discovery studio software package. The results of molecular docking supported the previous mechanism that Nitro group interacts with the 4-amino group of thiamine pyrophosphate and surrounding amino acids.

EXPERIMENTAL

All chemicals employed in this study were analytical reagents and solvents were distilled before use. The course of

the reactions was monitored by TLC and column chromatography purification was carried out using silica gel. Melting points were determined by XT-4 melting point apparatus. ¹H and ¹³C NMR spectrum was recorded on a Varian 400 NMR spectrometer at room temperature operating at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR by using DMSO-*d*₆ as solvent. Mass spectra were obtained at Bruker Daltonics APEX™ 4.7T ESI/MS. The *C. difficile* (ATCC 43593) and culture medium were purchased from China Center of Industrial Culture Collection.

General procedure of preparation of nitazoxanide analogues: To an ice cool solution of amino-heterocycle in distilled tetrahydrofuran (with 1.1 eq triethylamine) or pyridine was added an equimolar amount of benzoyl chloride with stirring. After the addition was complete, the reaction mixture was allowed to stand at room temperature and stirred overnight. The reaction was judged complete by TLC analysis. The crude product that separated on dilution was filtered, washed with 10 % NaHCO₃ solution, then several times with water. The pure compound was obtained by gradient column chromatography. While for circumstances which dilution does not give precipitate, then the mixture was extracted by EtOAc and purified by chromatography. Spectral data of novel compounds were listed as following.

N-(5-Chlorothiazol-2-yl)-2,4-difluorobenzamide (2a): Obtained in 56 % yield as white solid; m.p. 155–156 °C; ¹H-NMR (400 MHz, DMSO-*d*₆): δ 12.91 (s, 1H), 7.85 (td, 1H, *J* = 8.3, 6.5 Hz), 7.62 (s, 1H), 7.48 (ddd, 1H, *J* = 10.9, 9.5, 2.4 Hz), 7.32–7.22 (m, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.2 (dd, *J*_{CF} = 252, 12.3 Hz), 162.2, 160.2 (dd, *J*_{CF} = 255, 13.1 Hz), 155.7, 135.9, 132.3 (dd, *J*_{CF} = 10.7, 3.4 Hz), 118.7, 118.4 (dd, *J*_{CF} = 13.3, 3.5 Hz), 112 (dd, *J*_{CF} = 21.8, 3.5 Hz), 105 (t, *J*_{CF} = 26.1 Hz). HR-MS (ESI): Calcd. for C₁₀H₅N₂OSClF₂ (M + H): 274.9852; Found: 274.9846.

N-(5-Chlorothiazol-2-yl)-3,4-difluorobenzamide (2b): Obtained in 51 % yield as white solid; m.p. 196–197 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.99 (s, 1H), 8.18 (ddd, 1H, *J* = 11.5, 7.7, 2.1 Hz), 8.04–7.94 (m, 1H), 7.71–7.52 (m, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 163.3, 156.4, 152.2 (dd, *J*_{CF} = 253, 12.6 Hz), 149.2 (dd, *J*_{CF} = 247, 13 Hz), 135.8, 128.8, 126 (dd, *J*_{CF} = 7.5, 3.3 Hz), 118.7, 117.8 (t, *J*_{CF} = 19.3 Hz). HR-MS (ESI): Calcd. for C₁₀H₅N₂OSClF₂ (M + H): 274.9852; Found: 274.9849.

N-(5-Chlorothiazol-2-yl)-2-(trifluoromethyl)benzamide (2c): Obtained in 54 % yield as white solid; m.p. 207–208 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.10 (s, 1H), 7.89 (d, 1H, *J* = 7.5 Hz), 7.85–7.73 (m, 3H), 7.61 (s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 165.8, 155.6, 135.9, 132.9, 132.6, 131.1, 129, 126.5 (d, *J*_{CF} = 4.6 Hz), 126.2 (q, *J*_{CF} = 31.7 Hz), 123.5 (q, *J*_{CF} = 274 Hz), 118.8. HR-MS (ESI): Calcd. for C₁₁H₆N₂OSClF₃ (M + H): 306.9914; Found: 306.9920.

N-(5-Chlorothiazol-2-yl)-4-(trifluoromethyl)benzamide (2d): Obtained in 36 % yield as white solid; m.p. 189–190 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.18 (s, 1H), 8.27 (d, 2H, *J* = 8.1 Hz), 7.94 (d, 2H, *J* = 8.3 Hz), 7.66 (s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.4, 156.4, 135.9, 135.3, 132.3 (q, *J*_{CF} = 32.1 Hz), 129.2, 125.6 (d, *J*_{CF} = 3.7 Hz), 123.7 (q, *J*_{CF} = 273 Hz), 118.8. HR-MS (ESI): Calcd. for C₁₁H₆N₂OSClF₃ (M + H): 306.9914; Found: 306.9923.

N-(5-Bromothiazol-2-yl)-2,4-difluorobenzamide (3a):

Obtained in 68 % yield as white solid; m.p. 188-190 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.93 (s, 1H), 7.86 (td, 1H, *J* = 8.5, 6.5 Hz), 7.67 (s, 1H), 7.47 (ddd, 1H, *J* = 10.9, 9.5, 2.4 Hz), 7.30-7.23 (m, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.2 (dd, *J*_{CF} = 252, 12.4 Hz), 162.2, 160.2 (dd, *J*_{CF} = 255, 13.2 Hz), 158.1, 138.9, 132.3 (d, *J*_{CF} = 10 Hz), 118.4 (dd, *J*_{CF} = 13.4, 3.6 Hz), 112 (d, *J*_{CF} = 21.8 Hz), 105 (t, *J*_{CF} = 26.2 Hz), 102.3. HR-MS (ESI): Calcd. for C₁₀H₅N₂OSBrF₂ (M + H): 318.9347; Found: 318.9352.

N-(5-Bromothiazol-2-yl)-3,4-difluorobenzamide (3b):

Obtained in 60 % yield as white solid; m.p. 196-197 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.03 (s, 1H), 8.25-8.10 (m, 1H), 8.05-7.95 (m, 1H), 7.70-7.60 (m, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 163.3, 158.8, 152.3 (dd, *J*_{CF} = 253, 12.4 Hz), 149.2 (dd, *J*_{CF} = 247, 13.1 Hz), 138.9, 128.8, 126.1, 117.8 (t, *J*_{CF} = 18.5 Hz), 102.4. HR-MS (ESI): Calcd. for C₁₀H₅N₂OSBrF₂ (M + H): 318.9347; Found: 318.9348.

N-(5-Bromothiazol-2-yl)-2-(trifluoromethyl)benzamide (3c):

Obtained in 33 % yield as white solid; m.p. 211-213 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.11 (s, 1H), 7.89 (d, 1H, *J* = 7.5 Hz), 7.85-7.73 (m, 3H), 7.66 (s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 165.8, 158.0, 138.9, 132.8, 132.6, 131, 129, 126.5 (q, *J*_{CF} = 4.7 Hz), 126.2 (q, *J*_{CF} = 31.6 Hz), 123.5 (q, *J*_{CF} = 274 Hz), 102.4. HR-MS (ESI): Calcd. for C₁₁H₆N₂OSBrF₃ (M + H): 350.9409; Found: 350.9419.

N-(5-Bromothiazol-2-yl)-4-(trifluoromethyl)benzamide (3d):

Obtained in 49 % yield as white solid, m.p. 202-205 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.18 (s, 1H), 8.27 (d, 2H, *J* = 8.2 Hz), 7.94 (d, 2H, *J* = 8.2 Hz), 7.70 (s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.4, 158.8, 138.9, 135.3, 132.3 (q, *J*_{CF} = 32.1 Hz), 129.2, 125.5, 123.7 (q, *J*_{CF} = 271 Hz), 102.5. HR-MS (ESI): Calcd. for C₁₁H₆N₂OSBrF₃ (M + H): 350.9409; Found: 350.9412.

N-(5-Nitropyrimidin-2-yl)-2-(trifluoromethyl)benzamide (4c):

Obtained in 54 % yield as white solid; m.p. 158-160 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.09 (s, 1H), 9.40 (s, 2H), 7.86 (d, 1H, *J* = 7.8 Hz), 7.80-7.65 (m, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 166.1, 159.7, 154.8, 138.7, 135.2, 132.5, 130.4, 128.2, 126.4, 125.8 (q, *J*_{CF} = 31.5 Hz), 123.7 (q, *J*_{CF} = 274 Hz). HR-MS (ESI): Calcd. for C₁₂H₇N₄O₃F₃ (M + H): 313.0543; Found: 313.0551.

N-(5-Nitropyrimidin-2-yl)-4-(trifluoromethyl)benzamide (4d):

Obtained in 49 % yield as white solid; m.p. 140-143 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.04 (s, 1H), 9.49 (s, 2H), 8.16 (d, 2H, *J* = 8.1 Hz), 7.92 (d, 2H, *J* = 8.5 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.8, 160.5, 154.8, 138.7, 137.5, 132.1 (q, *J*_{CF} = 31.9 Hz), 129.5, 125.4, 123.8 (q, *J*_{CF} = 273 Hz). HR-MS (ESI): Calcd. for C₁₂H₇N₄O₃F₃ (M + H): 313.0543; Found: 313.0553.

N-(5-Chloropyrimidin-2-yl)-2,4-difluorobenzamide (5a):

Obtained in 31 % yield as white solid; m.p. 170-171 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.34 (s, 1H), 8.80 (s, 2H), 7.74 (td, 1H, *J* = 8.4, 6.6 Hz), 7.38 (ddd, 1H, *J* = 10.8, 9.5, 2.4 Hz), 7.24-7.17 (m, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 163.7 (dd, *J*_{CF} = 251, 12.4 Hz), 162.2, 159.8 (dd, *J*_{CF} = 252, 13.0 Hz), 156.7, 155.9, 132.0 (d, *J*_{CF} = 10.2 Hz), 125.1, 121 (dd, *J*_{CF} = 14.5, 3.6 Hz), 111.8 (d, *J*_{CF} = 21.6 Hz), 104.5 (t, *J*_{CF} = 26.0 Hz). HR-MS (ESI): Calcd. for C₁₁H₆N₃OCIF₂ (M + H): 270.0240; Found: 270.0238.

N-(5-Chloropyrimidin-2-yl)-3,4-difluorobenzamide (5b):

Obtained in 61 % yield as white solid; m.p. 149-150 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.34 (s, 1H), 8.87 (m, 2H), 8.04 (ddd, 1H, *J* = 11.4, 7.8, 2.2 Hz), 7.91-7.82 (m, 1H), 7.60 (dt, 1H, *J* = 10.4, 8.4 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 163.2, 156.7, 156.2, 151.9 (dd, *J*_{CF} = 252, 12.7 Hz), 149 (dd, *J*_{CF} = 247, 12.6 Hz), 131.3 (dd, *J*_{CF} = 4.8, 3.6 Hz), 126 (dd, *J*_{CF} = 7.6, 3.4 Hz), 125.3, 117.7 (dd, *J*_{CF} = 17.9, 14.9 Hz). HR-MS (ESI): Calcd. for C₁₁H₆N₃OCIF₂ (M + H): 270.0240; Found: 270.0241.

N-(5-Chloropyrimidin-2-yl)-2-(trifluoromethyl)benzamide (5c):

Obtained in 22 % yield as white solid; m.p. 216-218 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.50 (s, 1H), 8.76 (s, 2H), 7.83 (d, 1H, *J* = 7.7 Hz), 7.72 (dt, 2H, *J* = 14.6, 7 Hz), 7.63 (d, 1H, *J* = 7.5 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 165.8, 156.6, 155.7, 135.6, 132.4, 130.0, 128.2, 126.3 (q, *J*_{CF} = 4.6 Hz), 125.7 (q, *J*_{CF} = 31.4 Hz), 123.7, (q, *J*_{CF} = 274 Hz). HR-MS (ESI): Calcd. for C₁₂H₇N₃OCIF₃ (M + H): 302.0303; Found: 302.0309.

N-(5-Chloropyrimidin-2-yl)-4-(trifluoromethyl)benzamide (5d):

Obtained in 60 % yield as white solid; m.p. 170-171 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.49 (s, 1H), 8.87 (s, 2H), 8.14 (d, 2H, *J* = 8.6 Hz), 7.90 (d, 2H, *J* = 8.5 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.5, 156.7, 156.2, 137.9, 131.8 (q, *J*_{CF} = 31.9 Hz), 129.2, 125.3 (d, *J*_{CF} = 3.9 Hz), 123.8 (q, *J*_{CF} = 273 Hz). HR-MS (ESI): Calcd. for C₁₂H₇N₃OCIF₃ (M + H): 302.0303; Found: 302.0296.

N-(5-Bromopyrimidin-2-yl)-2,4-difluorobenzamide (6a):

Obtained in 19 % yield as white solid; m.p. 161-163 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.32 (s, 1H), 8.85 (s, 2H), 7.74 (td, 1H, *J* = 8.4, 6.7 Hz), 7.38 (ddd, 1H, *J* = 10.8, 9.5, 2.4 Hz), 7.21 (td, 1H, *J* = 8.5, 2.2 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 163.7 (dd, *J*_{CF} = 251, 12.4 Hz), 162.2, 159.8 (dd, *J*_{CF} = 252, 13 Hz), 158.8, 156.1, 132 (dd, *J*_{CF} = 10.5, 4.2 Hz), 121 (dd, *J*_{CF} = 14.4, 3.7 Hz), 113.8, 111.8 (dd, *J*_{CF} = 21.7, 3.5 Hz), 104.5 (t, *J*_{CF} = 26.3 Hz). HR-MS (ESI): Calcd. for C₁₁H₆N₃OBrF₂ (M + H): 313.9735; Found: 313.9737.

N-(5-Bromopyrimidin-2-yl)-3,4-difluorobenzamide (6b):

Obtained in 30 % yield as white solid; m.p. 173-174 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.30 (s, 1H), 8.91 (s, 1H), 8.04 (ddd, 1H, *J* = 11.5, 7.8, 2.2 Hz), 7.91-7.82 (m, 1H), 7.60 (dt, 1H, *J* = 10.5, 8.4 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 163.2, 158.7, 156.5, 151.9 (dd, *J*_{CF} = 252, 12.7 Hz), 149 (dd, *J*_{CF} = 247, 13 Hz), 131.3 (dd, *J*_{CF} = 4.9, 3.5 Hz), 125.9, 117.6, 114. HR-MS (ESI): Calcd. for C₁₁H₆N₃OBrF₂ (M + H): 313.9735; Found: 313.9739.

N-(5-Bromopyrimidin-2-yl)-2-(trifluoromethyl)benzamide (6c):

Obtained in 23 % yield as white solid; m.p. 192-193 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.47 (s, 1H), 8.81 (s, 2H), 7.82 (d, 1H, *J* = 7.5 Hz), 7.72 (dt, 2H, *J* = 15.2, 7.5 Hz), 7.63 (d, 1H, *J* = 7.5 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 165.8, 158.7, 156, 135.6, 132.4, 130, 128.2, 126.2, 125.7 (q, *J*_{CF} = 31.4 Hz), 123.7 (q, *J*_{CF} = 274 Hz), 113.8. HR-MS (ESI): Calcd. for C₁₂H₇N₃OBrF₃ (M + H): 345.9797; Found: 345.9792.

N-(5-Bromopyrimidin-2-yl)-4-(trifluoromethyl)benzamide (6d):

Obtained in 62 % yield as white solid; m.p. 180-182 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.45 (s, 1H), 8.93 (s, 2H), 8.14 (d, 2H, *J* = 8.2 Hz), 7.89 (d, 2H, *J* = 8.3 Hz); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 164.5, 158.8, 156.5, 137.9, 131.8

(q, $J_{\text{CF}} = 31.9$ Hz), 129.2, 125.3 (d, $J_{\text{CF}} = 3.7$ Hz), 123.8 (q, $J_{\text{CF}} = 273$ Hz), 114.1. HR-MS (ESI): Calcd. for $\text{C}_{12}\text{H}_7\text{N}_3\text{OBrF}_3$ (M + H): 345.9797; Found: 345.9801.

Determination of MIC values against *Clostridium difficile*: The antibacterial activities of synthesized compounds were tested by agar dilution method. *C. Difficile* (ATCC 43593) was incubated anaerobically overnight in chopped meat medium (anaerobe system) from stock and it was subcultured to a new chopped meat medium for 5 h at 37 °C. It was standardized to an optical density of 0.1 at OD600. Analogues were then diluted into the agar media at concentrations ranging from 0.125–16 µg/mL. Ten microliter volumes of the standardized inoculums were delivered to the surface of the agar plates. The plates were incubated for 18 h in an anaerobic chamber and were read visually for growth or no growth. Anaerobic plates containing no compound were used as controls and all experiments were performed 3 times in triplicate.

Molecular docking study of ligands with pyruvate ferredoxin oxidoreductase: The pdb file about the crystal structure of pyruvate ferredoxin oxidoreductase complex with thiamine pyrophosphate and pyruvate (2PDA) was obtained from the RCSB protein data bank (<http://www.pdb.org>). The molecular docking procedure was performed using CDOCKER protocol for receptor-ligand interactions section of discovery studio 3.5 (Accelrys Software Inc., San Diego, CA). In complex 2PDA, there were two same protein chains, one was deleted as well as inorganic ions. The ligands (nitazoxanide, **1d**, **2d**) and the enzyme pyruvate ferredoxin oxidoreductase were

prepared and optimized according to the default procedures. As described in literatures, pyruvate was thought to be binding to thiamine pyrophosphate and transformed into CO_2 , the interaction between pyruvate and pyruvate ferredoxin oxidoreductase was analyzed without any additional treatment. The binding site of molecular docking was settled around pyruvate with a radius of 10 Å. After the end of molecular docking, the pose with highest CDOCKER interaction energy (E_{cd}) was chosen as the most suitable pose and the types of interactions were analyzed. Finally, the interactions between ligands and pyruvate ferredoxin oxidoreductase were shown in 2D pictures.

RESULTS AND DISCUSSION

The synthesis of nitazoxanide analogues was accomplished according to Fig. 3. The starting compounds amino-heterocycles (head group analogues) and benzene ring derivatives (tail groups analogues) were commercially available or prepared by using common methods. The tail group and head group analogues were linked in anhydrous tetrahydrofuran with triethylamine acted as a base or in anhydrous pyridine. The final products were purified by gradient column chromatography. The yields of reactions were moderate since the strong electron-withdrawing effect of halogen substituents and nitro group. The structures of synthesized compounds were confirmed by ^1H NMR, ^{13}C NMR and HR-MS spectroscopy.

Antibacterial activities: As shown in Table-1, the antibacterial efficacy of synthesized compounds against *C. difficile* are expressed as minimum inhibitory concentration (MIC in

TABLE-1
MIC VALUES OF COMPOUNDS AGAINST *C. difficile*

1a-d, 2a-d, 3a-d					4c-d, 5a-d, 6a-d				
Compound	R ^[a]	X	MIC ^[b]	cLog P ^[c]	Compound	R ^[a]	X	MIC ^[b]	cLog P ^[c]
Nitazoxanide	2-OAc	NO ₂	0.25	2.02	4c	2-CF ₃	NO ₂	4	2.60
1a	2,4-2F	NO ₂	0.5	2.22	4d	2-CF ₃	NO ₂	2	2.64
1b	3,4-2F	NO ₂	1	2.22	5a	2,4-2F	Cl	>16	2.72
1c	2-CF ₃	NO ₂	0.5	2.81	5b	3,4-2F	Cl	>16	2.72
1d	4-CF ₃	NO ₂	0.125	2.86	5c	2-CF ₃	Cl	>16	3.32
2a	2,4-2F	Cl	>16	2.94	5d	4-CF ₃	Cl	>16	3.36
2b	3,4-2F	Cl	>16	2.94	6a	2,4-2F	Br	>16	2.86
2c	2-CF ₃	Cl	>16	3.53	6b	3,4-2F	Br	>16	2.86
2d	4-CF ₃	Cl	>16	3.58	6c	2-CF ₃	Br	>16	3.45
3a	2,4-2F	Br	>16	3.07	6d	4-CF ₃	Br	>16	3.50
3b	3,4-2F	Br	>16	3.07					
3c	2-CF ₃	Br	>16	3.66					
3d	4-CF ₃	Br	>16	3.71					

Compounds with high activities are shown in bold; ^[a]R=H, unless otherwise noted; ^[b]The MIC (µg/ml) values represent the mean of three experiments performed in triplicate, and the errors were within acceptable limits; ^[c]Calculated from <http://www.molinspiration.com>

Fig. 3 Synthesis of nitazoxanide analogues

$\mu\text{g/mL}$). The MIC value of compound **1d** is $0.125 \mu\text{g/mL}$, which is more potent than nitazoxanide ($0.25 \mu\text{g/mL}$). Other four compounds (**1b**, **1c**, **4d**, **4e**) containing nitro group exhibited moderate activities. The MIC values of compounds without nitro-group rank above $16 \mu\text{g/mL}$. These data suggested that the nitro group may be necessary for their outstanding antibacterial effects although amino-heterocycles can partially exert some influence. As for benzene ring, it is clear that compounds with *para* trifluoromethyl possess higher efficacy when compared with *ortho* substitution.

Molecular docking: In most anaerobes, the oxidative decarboxylation of pyruvate thereby form acetyl-coenzyme A is usually catalyzed by pyruvate ferredoxin oxidoreductase. Thus, it is a potential target for drug design against certain anaerobic pathogens. Besides, aiming to a better understanding of nitazoxanide's antibacterial activity and antiparasitic activity, the molecular docking study was attempted to prove the previous mechanism related to pyruvate ferredoxin oxidoreductase enzyme.

The complex 2PDA was obtained from RSCB protein data bank, which contains thiamine pyrophosphate, pyruvate and inorganic ions. It is a crystal structure of homodimeric desulfovibrio africanus pyruvate ferredoxin oxidoreductase and shows the noncovalent fixation of the substrate before the catalytic reaction²⁵. Without any processing, the interaction between pyruvate and pyruvate ferredoxin oxidoreductase was analyzed. The results displayed that thiamine pyrophosphate and

surrounding amino acid residues (ARG114, THR31) interacted with pyruvate. Then the pyruvate was removed and a binding site was set around pyruvate followed by docking study about different ligands nitazoxanide, **1d** and **2d**. The results gave by CDOCKER were analyzed and poses with the highest- E_{CD} were chosen as the most suitable cases. The docking results of nitazoxanide and **1d** suggested that the nitro group was interacted with thiamine pyrophosphate and the surrounding amino acids (ARG114, THR31, ASN996). Meanwhile, there was strong π - π interaction between the benzene ring and amino acid LYS459. While for compound **2d**, which the nitro group replaced by chlorine, the pose with highest E_{CD} show weak interactions with related amino acids, no interaction with thiamine pyrophosphate. Actually, the amino acids interacted with pyruvate were THR31, ARG114 and ASN996, which were same when nitazoxanide and **1d** set as ligand. The interactions between pyruvate ferredoxin oxidoreductase enzyme and ligands were shown in Figs. 4 and 5.

The interactions between nitazoxanide and pyruvate ferredoxin oxidoreductase were almost same compared with interactions between pyruvate and pyruvate ferredoxin oxidoreductase, which means that nitazoxanide may be a competitive inhibitor thus affect the energy metabolism in anaerobic organisms. Secondly, besides π - π interaction between benzene ring and amino acid LYS459, the interactions between pyruvate ferredoxin oxidoreductase and ligands were all related to nitro-group. This gives a reasonable explanation why compounds

Fig. 4. 2D pictures of interactions between pyruvate ferredoxin oxidoreductase and different ligands. A: Pyruvate; B: Nitazoxanide; C: **1d**; D: **2d**

Fig. 5. Binding mode of nitazoxanide and pyruvate ferredoxin oxidoreductase

without nitro-group show little antibacterial activities. Also, interactions between nitazoxanide and pyruvate ferredoxin oxidoreductase was related to the important nitro-group, rather than reported combination between nitazoxanide anion and thiamine pyrophosphate. So for the accurate mechanism of nitazoxanide's activities against anaerobic organisms, further studies are needed.

Conclusion

In conclusion, most synthesized nitazoxanide analogues were novel compounds (except **1a**, **1b**, **1c**, **1d**,) and structures were confirmed by ^1H NMR, ^{13}C NMR and HR-MS. Several compounds exhibited high or medium activity against *C. difficile* and the most promising compound was **1d**. While thiazole ring replaced by pyrimidine moiety, the efficacy was moderate. Molecular docking studies shown that ligands nitazoxanide and **1d** interacted with thiamine pyrophosphate and surrounding amino acid residues through nitro-group, which were almost same compared with interaction between pyruvate and pyruvate ferredoxin oxidoreductase. Thus, nitazoxanide may be a competitive inhibitor of pyruvate and nitro-group is crucial for the structure. To clarify the structure-activity relationship and mechanism of thiazolides' inhibition on anaerobic organisms, further studies are needed.

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REFERENCES

1. J.F. Rossignol and R. Cavier, *Chem. Abstr.*, **83**, 28216 (1975).
2. J.F. Rossignol and H. Maisonneuve, *Am. J. Trop. Med. Hyg.*, **33**, 511 (1984).
3. J.F. Rossignol, A. Ayoub and M.S. Ayers, *J. Infect. Dis.*, **184**, 103 (2001).
4. I.S. Adagu, D. Nolder, D.C. Warhurst and J.F. Rossignol, *J. Antimicrob. Chemother.*, **49**, 103 (2002).
5. D. Elvia, M. Jaime, R. Enrique and B. Rosamaria, *Am. J. Trop. Med. Hyg.*, **68**, 384 (2003).
6. H.M. Gilles and P.S. Hoffman, *Trends Parasitol.*, **18**, 95 (2002).
7. V.R. Anderson and M.P. Curran, *Drugs*, **67**, 1947 (2007).
8. C.S. McVay and R.D. Rolfe, *Antimicrob. Agents Chemother.*, **44**, 2254 (2000).
9. Y. Guttner, H.M. Windsor, C.H. Viala, L. Dusci and B.J. Marshall, *Antimicrob. Agents Chemother.*, **47**, 3780 (2003).
10. B.E. Korba, A.B. Montero, K. Farrar, K. Gaye, S. Mukerjee, M.S. Ayers and J.F. Rossignol, *Antiviral Res.*, **77**, 56 (2008).
11. J.F. Rossignol, S. La Frazia, L. Chiappa, A. Ciucci and M.G. Santoro, *J. Biol. Chem.*, **284**, 29798 (2009).
12. J.F. Rossignol, M. Abu-Zekry, A. Hussein and M.G. Santoro, *Lancet*, **368**, 124 (2006).
13. L.P. de Carvalho, C.M. Darby, K.Y. Rhee and C. Nathan, *ACS. Med. Chem. Lett.*, **2**, 849 (2011).
14. G. Sisson, A. Goodwin, A. Raudonikienė, N.J. Hughes, A.K. Mukhopadhyay, D.E. Berg and P.S. Hoffman, *Antimicrob. Agents Chemother.*, **46**, 2116 (2002).
15. P.S. Hoffman, G. Sisson, M.A. Croxen, K. Welch, W.D. Harman, N. Cremades and M.G. Morash, *Antimicrob. Agents Chemother.*, **51**, 868 (2007).
16. G.A. Pankuch and P.C. Appelbaum, *Antimicrob. Agents Chemother.*, **50**, 1112 (2006).
17. T.E. Ballard, X. Wang, I. Olekhovich, T. Koerner, C. Seymour, P.S. Hoffman and T.L. Macdonald, *Bioorg. Med. Chem. Lett.*, **20**, 3537 (2010).
18. T.E. Ballard, X. Wang, I. Olekhovich, T. Koerner, C. Seymour, J. Salamoun, M. Warthan, P.S. Hoffman and T.L. Macdonald, *Chem. Med. Chem.*, **6**, 362 (2011).
19. G. Navarrete-Vazquez, F. Chávez-Silva, R. Argotte-Ramos, M.C. Rodríguez-Gutiérrez, M.J. Chan-Bacab, R. Cedillo-Rivera, R. Moo-Puc and E. Hernández-Núñez, *Bioorg. Med. Chem. Lett.*, **21**, 3168 (2011).
20. M.J. Chan-Bacab, E. Hernandez-Nunez and G. Navarrete-Vazquez, *J. Antimicrob. Chemother.*, **63**, 1292 (2009).
21. A.V. Stachulski, C. Pidathala, E.C. Row, R. Sharma, N.G. Berry, M. Iqbal, J. Bentley, S.A. Allman, I.G. Edwards, A. Helm, J. Hellier, B.E. Korba, J.E. Semple and J.F. Rossignol, *J. Med. Chem.*, **54**, 4119 (2011).
22. A.V. Stachulski, C. Pidathala, E.C. Row, R. Sharma, N.G. Berry, A.S. Lawrenson, S.L. Moores, M. Iqbal, J. Bentley, S.A. Allman, G. Edwards, A. Helm, J. Hellier, B.E. Korba, J.E. Semple and J.-F. Rossignol, *J. Med. Chem.*, **54**, 8670 (2011).
23. M. Esposito, S. Moores, A. Naguleswaran, J. Müller and A. Hemphill, *Int. J. Parasitol.*, **37**, 1143 (2007).
24. J. Müller, G. Rühle, N. Müller, J.F. Rossignol and A. Hemphill, *Antimicrob. Agents Chemother.*, **50**, 162 (2006).
25. E. Chabrière, M.H. Charon, A. Volbeda, L. Pieulle, E.C. Hatchikian and J.C. Fontecilla-Camps, *Nat. Struct. Biol.*, **6**, 182 (1999).