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ORIGINAL ARTICLE

Synthesis, *in vitro* bioassays, and computational study of heteroaryl nitazoxanide analogs

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Abstract

Antiprotozoal drug nitazoxanide (NTZ) has shown diverse pharmacological properties and has appeared in several clinical trials. Herein we present the synthesis, characterization, in vitro biological investigation, and in silico study of four hetero aryl amide analogs of NTZ. Among the synthesized molecules, compound **2** and compound **4** exhibited promising antibacterial activity against *Escherichia coli* (*E. coli*), superior to that displayed by the parent drug nitazoxanide as revealed from the in vitro antibacterial assay. Compound **2** displayed zone of inhibition of 20 mm, twice as large as the parent drug NTZ (10 mm) in their least concentration (12.5 µg/ml). Compound **1** also showed antibacterial effect similar to that of nitazoxanide. The analogs were also tested for in vitro cytotoxic activity by employing cell counting kit-8 (CCK-8) assay technique in *HeLa* cell line, and compound **2** was identified as a potential anticancer agent having IC_{50} value of 172 µg which proves it to be more potent than nitazoxanide (IC_{50} = 428 µg). Furthermore, the compounds were subjected to molecular docking study against various bacterial and cancer signaling proteins. The in vitro test results corroborated with the in silico docking study as compound **2** and compound **4** had comparatively stronger binding affinity against the proteins and showed a higher docking score than nitazoxanide toward human mitogen-activated protein kinase (MAPK9) and fatty acid biosynthesis enzyme (FabH) of *E. coli*. Moreover, the docking study demonstrated dihydrofolate reductase (DHFR) and thymidylate synthase (TS) as probable new targets for nitazoxanide and its synthetic analogs. Overall, the study suggests that nitazoxanide and its analogs can be a potential lead compound in the drug development.

KEYWORDS
cytotoxicity assay, DHFR, *E. coli*, MAPK9, nitazoxanide, TS

1 | INTRODUCTION

Nitazoxanide (NTZ) or 2-acetyloxy-N-(5-nitro-2-thiazolyl) ben-zamide (Figure 1) belongs to 5-nitrothiazole group of molecules which has already received ample attention in the field of drug dis-covery and drug development.[1,2] NTZ possessed broad spectrum of activity against protozoa,[3,4] helminthes,[5,6] and numerous Gram-positive and Gram-negative anaerobic bacteria.[6–8] NTZ also exhib-ited potential antiviral properties[9,10] and, recently, it has been found to be effective against SARS-CoV-2[11] and a number of clinical trials are underway.[12,13]

Because of the potential biological properties, structurally mod-ified NTZ-based analogs or structurally related molecules were syn-thesized and investigated for their biological activity. Some recently synthesized analogs of NTZ reportedly possess prominent antipro-tozoal,[14–17] antibacterial, and antimycobacterial[18,19] and antiviral[20] activities even in some cases better than the parent drug NTZ itself. Hence, NTZ became the drug of interest for our research where we focused on synthesis of hetero-aryl amide analogs.

Although, NTZ and some of its analogs were investigated for a wide range of biological activities, heteroaryl amide analogs were not screened for several bioactivities. Therefore, we planned to synthesize some heteroaryl amide analogs (Figure 1) to evaluate their diverse bi-ological activities such as antibacterial, antiviral, anticancer activity, in order to prove them to be a potential therapeutic choice. Based on the in vitro biological activities, we also conducted in silico molecular dock-ing study to understand a mechanistic insight regarding the activity of NTZ and its synthetic analogs. So far, we have investigated in vitro anti-bacterial, cytotoxic and in vivo analgesic and anti-inflammatory effects of NTZ and four synthesized analogs. Although, the analogs did not produce significant analgesic and anti-inflammatory effects, we found some interesting results in antibacterial and cytotoxic screening which we are going to disclose in this paper.

2 | MATERIALS AND METHODS

2.1 | General experimental procedures

All the synthetic procedures were conducted in Chemical Biology and DNA Synthesis Laboratory, Faculty of Pharmacy, University of Dhaka. The solvents were dried and properly distilled. Progress of the reactions was monitored by Thin Layer Chromatography (TLC), and visualization was accomplished by using UV light at 254 nm. For column chromatography, silica gel 60 (0.06–0.2 mm, ROTH) was employed. ^{1}H NMR (400 MHz) was recorded on Ultra Shield Bruker 400 NMR instrument, using DMS0-d6, and the chemical shifts are reported as δ (ppm) with respect to tetramethylsilane (TMS) (ppm) as internal standard. Fourier-transform infrared (FT-IR) Spectra were recorded with FT-IR 8400S Shimadzu spectrophotometer in the range of 4000–400 cm^{-1} using KBr pressed pellet technique. All the reagents used in synthetic procedure were purchased from Sigma-Aldrich, Germany. Nitazoxanide, metronidazole, and nalidixic acid were procured from Incepta Pharmaceuticals Ltd. as dried powder.

2.2 | Synthesis

Triethylamine (TEA) (1.2 equiv.) was added to the solution of 2-amino-5-nitrothiazole (0.00689 mol) and dichloromethane (DCM). After the mixture was stirred for 15 min at 5°C, a solution of hetero aryl acid chloride (1.1 equiv.) in dichloromethane was added dropwise. The reaction mixture was stirred at room temperature for 24 h and after the completion of reaction indicated by TLC, the resulting residue was neu-tralized by saturated NaHCO$_3$ solution. The mixture was then extracted with ethyl acetate. The organic layer was washed with brine solution. The solvent was removed under reduced pressure and crude product was then subjected to column chromatography for purification.

2.3 | Spectrometric characterization of synthesized compounds

The structure of the synthetic analogs of nitazoxanide was eluci-dated spectroscopically by using IR and ^{1}H NMR and by comparing the data with that of the reported data.[17,21] IR and ^{1}H-NMR data are recorded as follows:

2.3.1 | N-(5-nitrothiazol-2-yl) thiophene-2-carboxamide (compound 1)

Yield: 91%; light yellow solid; R$_f$: 0.77 (ethyl acetate: n-hexane = 1:2); IR (KBr, cm^{-1}): 1542 (Aryl C=C str), 3077 (Aryl C–H str), 1265 (C–N
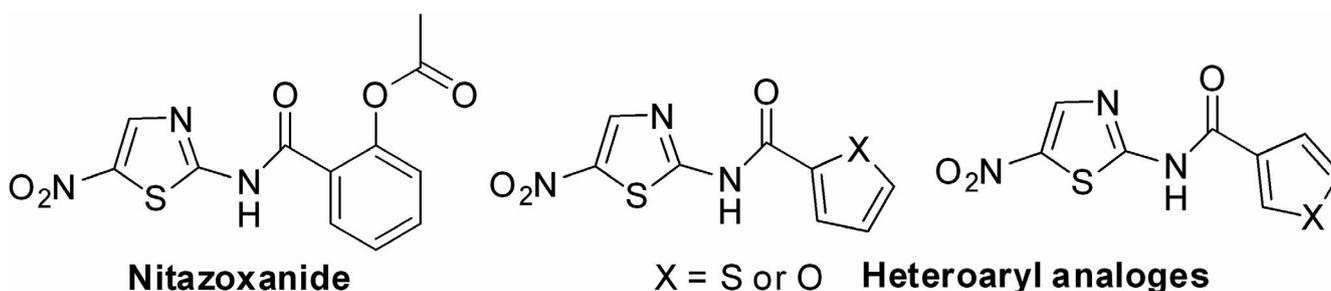

FIGURE 1 Structure of NTZ and heteroaryl analogs

str thiazole), 1351, 1542 (N–O str), 1662 (C=O str amide), 3350 (N–H str amide); ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 8.69 (s, 1H), 8.29 (d, J = 3.2 Hz, 1H), 8.05 (d, J = 4.8 Hz, 1H), 7.27 (dd, J = 3.2, 4.8 Hz, 1H).

2.3.2 | *N*-(5-nitrothiazol-2-yl) thiophene-3-carboxamide (compound 2)

Yield: 89%; yellow solid; R$_f$: 0.54 (ethyl acetate: n-hexane = 1:2); IR (KBr, cm^{-1}): 1548 (Aryl C=C str), 3103 (Aryl C–H str), 1317 (C–N str thiazole), 1354, 1548 (N–O str), 1674 (C=O str), 3120 (N–H str amide); ^{1}H NMR (400 MHz, DMSO-d$_6$): 8.70 (s, 1H), 8.69 (s, 1H), 7.74 (d, J = 10 Hz, 1H), 7.72 (d, J = 10 Hz, 1H).

2.3.3 | *N*-(5-nitrothiazol-2-yl) furan-2-carboxamide (compound 3)

Yield: 70%; beige solid; R$_f$: 0.90 (ethyl acetate: n-hexane = 1:2); IR (KBr, cm^{-1}): 1411 (Aryl C=C str), 3143 (Aryl C–H str), 1315 (C–N str thiazole), 1356, 1482 (N–O str), 1667(C=O stretching of amide functional group), 3350 (N–H stretching of amide group); ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.77 (s, 1H), 7.72 (br s, 1H), 7.32 (br s, 1H), 7.06 (s, 1H), 6.57 (d, J = 1.6 Hz, 1H).

2.3.4 | *N*-(5-nitrothiazol-2-yl) furan-3-carboxamide (compound 4)

Yield: 53%; yellow solid; R$_f$: 0.96 (ethyl acetate: n-hexane = 1:2); IR (KBr, cm^{-1}): 1313 (Aryl C=C str), 3139 (Aryl C–H str), 1313 (C–N str thiazole) 1355, 1507 (N–O str), 1653 (C=O stretching of amide group), 3446 (N–H stretching of amide group); ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 8.67 (s,1H), 8.65 (s,1H), 7.86 (s,1H), 7.10 (s,1H).

2.4 | Antibacterial activity assay against *S. aureus* and *E. coli*

NTZ and its four analogs were assayed for antibacterial activity against a gram-positive *Staphylococcus aureus* (*S. aureus* – coagulase (+) ve ATCC: 20121107-4) and a gram-negative (*E. coli* ATCC: 0157-CR3) strain by the standardized disc diffusion method.[22] All the test samples were dissolved in DMSO (0.1% v/v) and diluted to prepare four concentrations (100, 50, 25, and 12.5 µg/ml) for each test sample. Nalidixic acid was used as the standard drug. The zone of inhibition was compared with standard drug after 24 h of incubation at 37°C for antibacterial activity.

2.5 | Cytotoxicity assay

HeLa, a human cervical carcinoma cell line, was used for the cytotoxicity assay. Here the assay was designed in two phases. Firstly,

the quantity of cell viability was determined by Trypan blue dye exclusion technique.[23] Then MTT method was performed to determine the IC$_{50}$ value (50% growth inhibitory concentration) from the calculation of percent growth inhibition.

2.5.1 | Trypan blue dye exclusion method

A cell suspension was made with a fixed volume of cells (e.g. 1 ml) and 50 µl of cell suspension was taken in a cryo vial. Equal parts of 0.4% trypan blue dye were added to the cell suspension and mixed them well by pipetting up and down. The cell suspension was then transferred to a hemocytometer to count the live cell and dead cell. Viable cell will resist the dye to enter as it possesses intact cell membrane whereas dead cell will take up the dye and turn into blue staining. Typically, each side of the hemocytometer will take 10–20 µl of the solution. After that the hemocytometer was placed on the stage of an inverted microscope. All the cells (clear and blue) in each large square in each corner of the hemacytometer were counted. Blue cells were the non-viable cells and clear cells were the viable cells. Calculation of the percentage of viable cells was done by using the following formula: Percentage (%) cell viability = (Live cell count/ Total cell count) × 100.

2.5.2 | MTT assay

Hela cell line was cultured in DMEM (Dulbecco's Modified Eagles' medium) containing 1% penicillin–streptomycin (1:1), 0.2% gentamycin, and 10% fetal bovine serum (FBS) and was incubated at 37°C with an atmosphere of 5% CO$_2$. Cell Counting Kit-8 (CCK-8), a non-radioactive colorimetric cell proliferation and cytotoxic assay kit (Sigma-Aldrich), was employed for the in vitro cytotoxicity test.[24,25] Cells were seeded onto 96-well plates at a concentration of (2 × 10^4/100 µl) and incubated at 37°C with an atmosphere of 5% CO$_2$ for 24 h. Each sample measuring 25 µl (filtered) was added into each well in duplicate. Positive control (NTZ) and compounds **2** and **4** (500 and 100 µg/ml) were dissolved in DMSO (0.1% v/v). Cells were periodically checked for granularity, shrinkage, and swelling using trinocular microscope with camera (Optika) during the incubation period of 48 h. After incubation, 10 µl of CCK-8 (5 mg/ml) solution was added to each well followed by incubation at 37°C for 4 h for cytotoxicity. The viable cells were visualized by the presence of purple color formazan dye. As the amount of produced formazan dye is directly proportional to the number of living cells, the measurement of absorbance value will give the number of viable cells.[23,24] The absorbance values were measured using a microplate reader at 570 nm wavelength where DMSO was used as blank. The percentage growth inhibition was calculated using the following formula.[26]

$$\% \, \text{cell inhibition} = \left\{ \left(A_t - A_b \right) / \left(A_c - A_b \right) \right\} \times 100$$

where, A_t = Mean absorbance value of test compound, A_b = Mean absorbance value of blank, A_c = Mean absorbance value of control.

Percentage of inhibition is plotted against the drug concentration, and IC_{50} value is determined.

2.6 | Molecular docking study

The current protocol followed 'rigid ligand–rigid receptor' docking. The crystallographic structures of the selected target proteins were downloaded from RCSB Protein Data Bank (RCSB-PDB)[27] in PDB format. The ligands were drawn in ChemBioDraw Ultra 12.0 and then collected in SDF format from the PubChem[28] database. Nitazoxanide's accession no. in Drug Bank database, DB00507,[29] CASTp,[30] and DoGSiteScorer[31] were used to predict the active sites of the target proteins. The structures of the target proteins were converted into PDBQT format after performing necessary modifications in AutoDoc Tools (ADT) (version 1.5.6).[32] The ligands were also converted into PDBQT format with Open Bable tool.[33] Finally, the docking of the ligands with the target proteins was done by using the Autodock Vina (version 1.1.2).[34]

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology. org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[35] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[36]

3 | RESULTS AND DISCUSSION
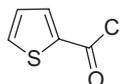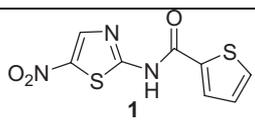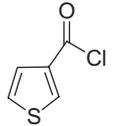
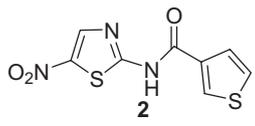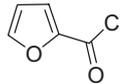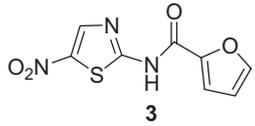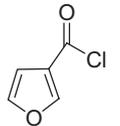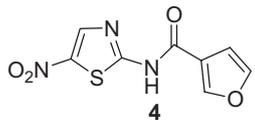## 3.1 | Synthesis of heteroaryl amide derivatives

In the present work, 2-amino-5-nitrothiazole has been used as the starting material which was conjugated with various hetero arylchloride using well known Schotten Baumann[37,38] reaction in the presence of triethylamine (TEA) and dichloromethane (DCM) to produce the hetero aryl amide analogs of NTZ (Table 1). During synthesis of different analogs, 5-nitro thiazole group was kept constant as drug activation largely depends on the redox potential of the 5-nitro group, because reduction of 5-nitro group by nitroreductases, including pyruvate ferredoxin oxidoreductase (PFOR), is responsible for NTZ's mechanistic activation and, likewise, spectrum of activity.[39] Moreover, heterocyclic rings (e.g. thiophene and furan) were incorporated replacing the benzene ring in the synthetic analogs to understand the role of hetero aryl residues.

According to the synthetic method described above, four nitazoxanide analogs have been synthesized (Table 1). The analogs designated as compound **1, 2, 3** and **4** were produced having a good yield 91%, 89%, 70%, and 53%, respectively. Structures of the

TABLE 1 Synthesis of heteroaryl amide derivatives as NTZ analogues
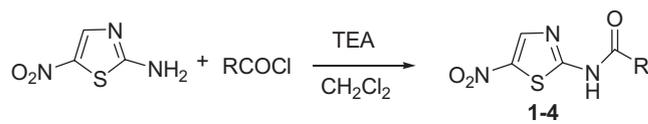
Entry	RCOCl	Amides	Yield%
1		**1**	91
2		**2**	89
3		**3**	70
4		**4**	53

synthesized compounds were elucidated by analyses of their high resolution ^{1}H-NMR and FT-IR spectroscopic data and were further confirmed by comparing their spectral data to that of the published values.[17,21] The spectral features are in close agreement with the published data.

3.2 | Antibacterial activity assay against *S. aureus* and *E. coli*

In vitro antibacterial effect of the four NTZ analogs on *E. coli* and *S. aureus* was observed on the basis of differential concentration of the test samples. *E. coli* was found to be more sensitive toward the synthetic compounds as most of the samples exhibited zone of inhibition in the range of 10–22 mm (Table 2). By contrast, *S. aureus* was rather resistant toward NTZ and the synthetic compounds as most of them did not show notable zone of inhibition (6 mm). Noticeably, only compound **1** and compound **3**, both at a concentration of 100 µg/ml, showed moderate zone of inhibition.

Compound **2** exhibited greatest activity among all the synthetic compounds including NTZ in all concentrations. This compound displayed zone of inhibition 20 mm which was twice as large as the parent drug NTZ (10 mm). The observed antibacterial effect against *E. Coli* was similar or superior to the standard Nalidixic acid. Compound **4** also showed remarkable activity by displaying larger zone of inhibition than the parent drug NTZ in all concentrations. The activity of Compound **1** and **3** was also moderate and comparable to that

TABLE 2 Antibacterial activity assay against *Escherichia coli* and *Staphylococcus aureus* in differential concentration

Test samples	Zone of inhibition (mm)[a]							
	Concentration (µg/ml)							
	100	50	25	12.5	100	50	25	12.5
	E. coli				*S. aureus*			
1	15	14	14	6	10	6	6	6
2	22	21	20	20	6	6	6	6
3	14	10	6	6	12	9	6	6
4	16	13	13	13	6	6	6	6
NTZ	15	12	12	10	6	6	6	6

[a]Nalidixic acid (30 µg/disc): 20 mm in *E. coli* and 19 mm in *S. aureu*.

TABLE 3 Trypan blue dye exclusion test to determine the percentage of cell viability

Compound	Concentration µg/ml	Live cell count	% cell viability
Control	–	2×10^4	100%
DMSO	–	19×10^3	>95%
1	20	19×10^3	>95%
	100	19×10^3	>95%
	500	12×10^3–14×10^3	60%-70%
2	20	19×10^3	>95%
	100	11×10^3–12×10^3	50%-60%
	500	6×10^3–8×10^3	30%-40%
3	20	19×10^3	>95%
	100	19×10^3	>95%
	500	19×10^3	>95%%
4	20	19×10^3	>95%
	100	19×10^3	>95%
	500	9×10^3–11×10^3	45%-55%
NTZ	20	19×10^3	>95%
	100	18×10^3	92%
	500	8×10^3–10×10^3	40%-50%

of NTZ. The present results indicate that hetero aryl NTZ analogs might be more potent antimicrobial agent.

3.3 | Trypan blue dye exclusion test for cytotoxicity

Trypan blue dye exclusion test is a widely used technique for determining the number of viable cells present in a cell suspension. It is based on the principle that viable cells possess intact cell membranes that is impermeable to the dyes such as trypan blue. On the other hand, dead cells will easily take up the dye and get blue staining. Thus, the viable cells can be easily distinguished from the dead cells as the viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm under a microscope.[23] According to the

procedure stated above, percentage of the viable cells was determined and the results are summarized in Table 3. It is clear that the % cell viability decreased with increasing concentration of test compounds. Among all of the synthetic compounds, **2** and **4** exhibited the highest growth inhibition (30%-40% and 45%–55%, respectively at 500 µg/ml concentration) which were similar or superior to that obtained by NTZ (40%–50% inhibition at the same concentration).

3.4 | MTT assay for cytotoxicity

Based on the result of the trypan blue dye exclusion test, compound **2** and compound **4** were further subjected to MTT assay for determination of their cytotoxic potentials as these two derivatives along with the parent NTZ demonstrated less than 50% cell viability. In the MTT assay, compound **2** demonstrated the highest growth inhibition with an IC_{50} value 172 µg which is superior to that exhibited by. NTZ (IC_{50} = 498 µg) (Table 4). This result indicates that compound **2** may act as a potential anticancer agent and proved it to be more potent than nitazoxanide. Compound **4** manifested moderate cytotoxic activity (IC_{50} = 490 µg) Recently, some studies have proposed that NTZ might exhibit anticancer activity in ovarian[40] and colorectal cancer[40–42] by means of interfering various crucial metabolic and pro-death signaling mechanisms such as drug detoxification, unfolded protein response (UPR), autophagy, anti-cytokines activities, and c-Myc inhibition.[43] These reports justify our in vitro cytotoxic test results. It is the very first report of in vitro cytotoxic activity of the synthesized NTZ analogs.

3.5 | Molecular docking

For better understanding the impact of NTZ and its heteroaryl analogs on the above biological activities, the molecular docking study was performed. The probable target proteins chosen for the current docking simulation study were those which are somewhat involved in the biochemical pathways regarding their respective biological activities. For understanding anticancer activity, we targeted some cancer signaling proteins, for example thymidylate synthase (TS),

TABLE 4 Cytotoxicity of the synthesized compounds

Name of the sample	Concentration (µg/ml)	Mean absorbance at 570 nm	% cell viability	% growth inhibition	IC$_{50}$ µg
Blank (DMSO and Media)	—	0.0515	—	—	—
Control (Cells with DMSO)	0	0.4445	100	0	—
Compound **2**	500	0.1860	34.23	65.77	172
	100	0.2615	53.44	46.56	
Compound **4**	500	0.2460	49.50	50.50	490
	100	0.3275	70.23	29.77	
NTZ	500	0.2126	41.01	58.99	428
	100	0.4087	90.89	9.11	

TABLE 5 Result of molecular docking of the compounds against 14 target proteins

| Protein	PDB ID	Organism	UniProt ID	Active site	Affinity (kcal/mol) with				
					NTZ	1	2	3	4
PDH	2IEA	*E. coli*	—	Pocket prediction (whole)	−9.1	−7.3	−7.5	−8.1	−7.4
PFOR	2C42	*D. africanus*	P94692	31T, 64E, 114R, 996N	−9	−7	−8.1	−7.6	−8.2
G6PS	2J6H	*E. coli*	P17169	2C, 604K	−8.3	−7.7	−7.2	−7.8	−7.2
MAPK8	3O17	*H. sapiens*	P45983	151D	−7.9	−7.2	−6.5	−7.4	−6.8
MAPK9	3E7O	*H. sapiens*	P45984	151D	−7	−5.3	−7.9	−5.6	−8.8
MAPK10	4KKE	*H. sapiens*	P53779	189D	−7.9	−6.3	−6.4	−6.2	−6.7
DHFR	4M6J	*H. sapiens*	P00374	NADPH binding site	−7.7	−6.4	−7.2	−6.4	−7.5
GSTP1	17GS	*H. sapiens*	P09211	8Y, 39W, 45K	−7.3	−5.3	−6.1	−5.8	−6.2
TS	4UP1	*H. sapiens*	P04818	195C	−7.1	−5.9	−5.9	−6	−6.3
mTORC1	5H64	*H. sapiens*	P42345	2340H	−6.7	−5.9	−6	−6	−6.1
FabH	3IL9	*E. coli*	P0A6R0	Pocket prediction (DogSiteScorer)	−5.2	−4.7	−5.7	−5.2	−6.3
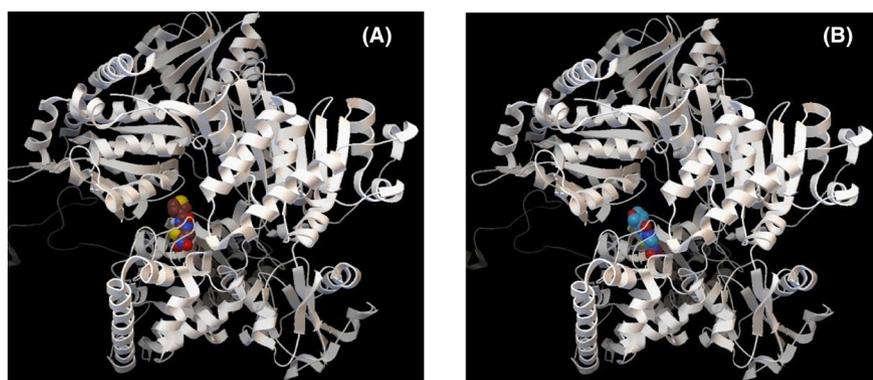
FIGURE 2 Binding Interaction of Synthesized analogs with PFOR. (A) Compound **2** and PFOR, (B) Compound **4** and PFOR

dihydrofolate reductase (DHFR), mechanistic target of Rapamycin complex 1 (mTORC1), mitogen activated kinase 8, 9 and 10 (MAPK 8, MAPK9, MAPK10), etc., and for investigating antibacterial activity, some bacterial proteins for example pyruvate ferredoxin oxidoreductase (PFOR), pyruvate dehydrogenase (PDH), β-ketoacyl-acyl carrier protein synthase III (FabH), and glucosamine-6-phosphate synthase (G6PS) were chosen. All the interactions having RMSD (Root Mean Square Deviation) value 0 indicates a good docking prediction.[44]

In case of interaction with the bacterial enzymes for example PFOR, PDH, G6PS, and FabH, the compounds showed a

remarkable binding affinity (−4.7 to −8.2 kcal/mol) toward them (Table 5). For instance, in case of PFOR, all the compounds along with NTZ showed an excellent interaction (−7 to −9 kcal/mol) with Thr31, Glu64, Arg114, and Asp996, the active site of PFOR. NTZ demonstrates its bioactivity by blocking PFOR, an essential enzyme for energy metabolism in anaerobes, leading to bacterial and protozoal death.[45,46] Among the synthetic compounds, **2** and **4** showed better interaction than the other compounds (Figure 2). This computational finding is in consistence with a previous study by Scior,[15] where a notable binding affinity was reported by the author between their synthetic NTZ analogs and PFOR. Like PFOR, pyruvate dehydrogenase (PDH) also plays the same role in glucose consumption and energy metabolism via oxidation of pyruvate in aerobic organism (e.g. *E. coli*) and mammals.[17] NTZ had been reported to inhibit 35 to 80% growth of *E. coli* in a dose-dependent manner by inhibiting PDH.[46] This fact is well supported by our excellent docking score ranging from −7 to −9.1 kcal/mol, for NTZ and the analogs toward PDH (Table 5, Figure 3A and B). Another enzyme glucosamine-6-phosphate synthase (G6PS) is required for building cell wall macromolecules like chitin and mannoproteins in fungi and peptidoglycans in bacteria, via the production of a molecule named uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc).[47] Recently G6PS has been targeted for in silico study involving substituted thiazoles that showed significant binding affinity toward the enzyme[47] as is evident in current docking experiment where all the compounds including NTZ presented strong interaction (−7.2 to −8.3 kcal/mol) with G6PS in its active site Cys2 and Lys604. NTZ and its analogs showed moderate (−4.7 to −6.3 kcal/mol) affinity with FabH, a key enzyme in the fatty acid biosynthesis pathway (FAB) of prokaryotes which gets inhibited by metronidazole-thiazole derivative in its active site.[48] Here, Compound **2** (−5.7 kcal/mol) and compound **4** (−6.3 kcal/mol) had greater docking score than NTZ (−5.2 kcal/mol) against FabH.

Furthermore, NTZ and all the synthesized compounds were examined for their interaction with various cancer signaling proteins including MAPK9 (Figure 3C and D), DHFR (Figure 3E and F), and TS (Figure 3G and H). All the synthesized compounds were found to have prominent binding affinity (−5.3 to −8.8 kcal/mol) in case of interaction with various cancer signaling proteins for example DHFR, TS, mTORC1, GSTP1, MAPK8, MAPK9, and MAPK10 (Table 5). For instance, MAPK 8, MAPK 9, and MAPK10 were identified as potential cellular targets for NTZ in a recent in silico study.[49] All compounds exhibited a favorable interaction (−5.3 to −8.8 kcal/mol) at their active sites; Asp151 in MAPK 8, 9 and with Asp189 in MAPK10. Among the synthetic analogs, compound **2** and compound **4** (Figure 3) showed stronger affinity than NTZ toward MAPK9.

Another two cancer signaling proteins, glutathione-S-transferase of the Pi class (GSTPI)[50] and mechanistic target of rapamycin complex 1 (mTORC1)[40,43] have been reported to be a promising mammalian target for NTZ. NTZ inhibits these proteins and causes cancer cell death in colorectal and ovarian cancer.[40,41,50] These reports support our docking finding where the compounds seemed to have
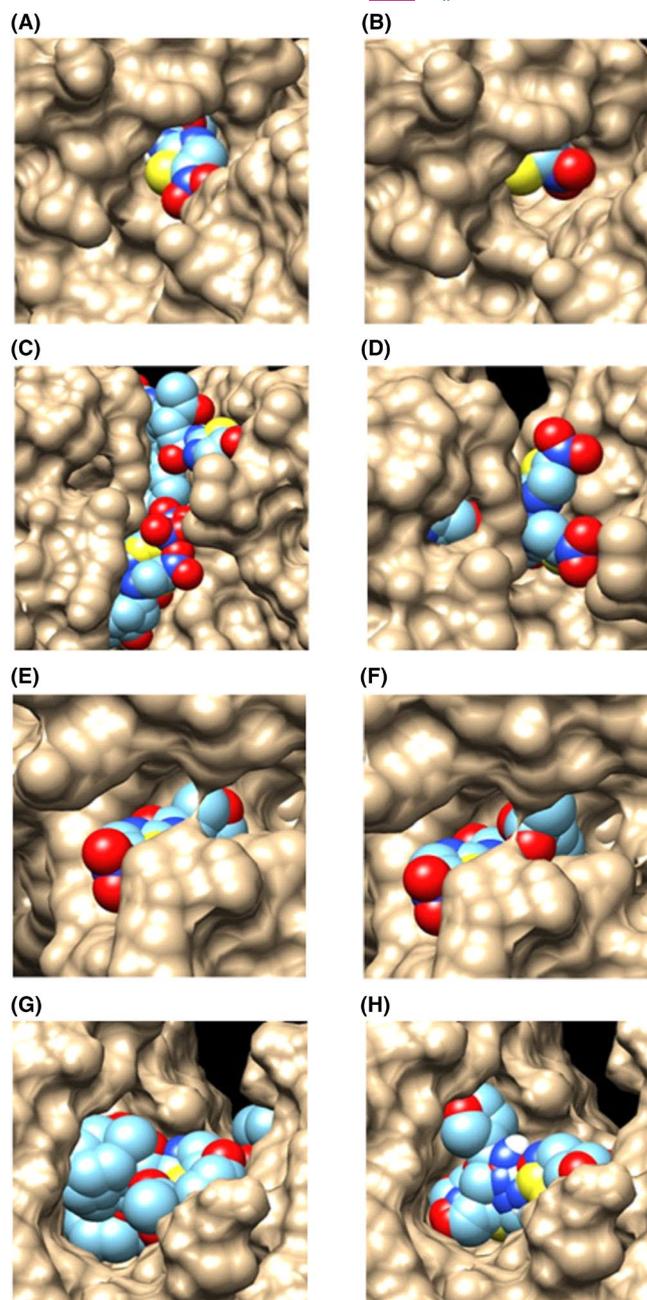

FIGURE 3 Graphical representation of the interaction of NTZ (multicolor) and the synthesized compounds (multicolor) with the respective proteins (brown) obtained by docking study. (A) Binding mode of NTZ with active site of PDH. (B) Binding mode of Compound **3** with active site of PDH. (C) Binding mode of NTZ with active site of MAPK9. (D) Binding mode of Compound **4** with active site of MAPK9. (E) Binding mode of NTZ with active site of DHFR. (F) Binding mode of Compound **4** with active site of DHFR. (G) Binding mode of NTZ with active site of TS. (H) Binding mode of Compound **4** with active site of TS. Abbreviations: DHFR, Dihydrofolate reductase; MAPK9, Mitogen activated kinase 9; NTZ, Nitazoxanide; PDH, Pyruvate dehydrogenase; TS, Thymidylate synthase

considerable affinity toward GSTPI (−5.3 to −7.3 kcal/mol) in its active site Tyr8, Trp39, Lys45, and mTORC1 (−5.9 to −6.7 kcal/mol) in its active site His2350.

Interestingly, the synthesized analogs and NTZ also displayed a notable interaction (−6.4 to −7.7 kcal/mol) with DHFR in its NADPH binding site and with TS (−5.9 to −7.1 kcal/mol) in its active site Cys195 (Figure 3). These two enzymes are essential for cell proliferation and cell growth,[51] and inhibition of them causes disruption of DNA synthesis and cell death consequently.[52,53] Therefore, the mentioned molecular affinity of the compounds toward DHFR and TS makes them a potential novel target for NTZ and its synthetic analogs in anticancer therapy.

From the docking scores provided in Table 5, some important inferences can be made. For instance, NTZ and the synthesized analogs showed excellent binding affinity with all the proteins except FaBH. Additionally, NTZ and the synthesized analogs revealed potential binding affinity with TS and DHFR. To the best of our knowledge, till date NTZ and analogs have never been subjected to molecular docking study against TS and DHFR, which postulates them as novel targets for NTZ. Moreover, the synthesized compounds have never encountered any molecular docking study against the mentioned proteins previously, and the docking score reveals their potential of having new pharmacological activities.

In most of the cases, compound **2** and **4** accounted for stronger binding affinity with the target proteins than do the other two compounds (Table 5). In fact, compound **2** and compound **4** showed better affinity than the parent drug NTZ against MAPK9 and FabH. These observations further reinforce both the in vitro antibacterial and cytotoxicity test results, where compound **2** and **4** exhibited greater inhibitory activity than the other compounds. Also compound **2** displayed larger zone of inhibition and proved to be more cytotoxic in cancer cell line, compared with NTZ. Thus, an important correlation can be made between the docking prediction and biological activity which is well supported by the previous studies that elucidated the role of NTZ as antibacterial and anticancer agent by blocking its essential metabolic process and interfering in multiple cancer signaling pathways, respectively.

4 | CONCLUSION

We have synthesized four heteroaryl analogs of NTZ by condensing 2-amino-5-nitrothiazole with some acid chlorides. Among the four synthesized analogs of NTZ, compound **2** and compound **4** displayed better antibacterial activity than NTZ against *E. coli*. Additionally, cytotoxicity assay in *HeLa* cell line demonstrated greater cell growth inhibition for compound **2,** which was proved to be more potent than NTZ. This is the very first report of cytotoxic as well as antibacterial activities of these synthesized compounds. The results of in vitro bioassays further corroborated with the molecular docking study that helped exploring probable mechanistic insights underlying their bioactivity. Moreover, the computational investigation identified DHFR and TS as potential novel targets for NTZ and its synthesized analogs. Although further detail experiments are warranted, these findings are unique and hence should draw attentions of structural, as well as chemical biologists, for future drug development considering nitazoxanide along with its analogs for repurposing it as a potential anticancer agent besides its conventional antibacterial and antiprotozoal uses. In addition, the analogs might be effective as antiviral agent against SARS-CoV-2 and, therefore, synthesis of new series of molecules followed by antibacterial, antiviral, and cytotoxicity assays will be focused in the future work. In addition, our future study will also focus on the in vivo and biochemical investigation of the synthesized analogs.

DISCLOSURE

None.

AUTHOR CONTRIBUTIONS

Rahman, S. M. A. and Chowdhury, A. K. A. conceptualized, designed, and supervised the research work. Ahmed, T. performed the synthesis and biological work. Asaduzzaman, M and Islam A. B. M. K performed molecular docking studies. Asaduzzaman M and Ahmed T did the data analysis. Rahman, S. M. A., Ahmed, T. and Asaduzzaman, M. wrote the manuscript. All authors reviewed the manuscript and approved it.

DATA AVAILABILITY STATEMENT

The data that support these results are available from the corresponding author upon reasonable request.

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