Bull. Chem. Soc. Ethiop. **2023**, 37(2), 373-389.
DOI: https://dx.doi.org/10.4314/bcse.v37i2.10

ISSN 1011-3924
Printed in Ethiopia
Online ISSN 1726-801X

SYNTHESIS OF SOME NEW THIOPHENE-BASED COMPOUNDS AND EVALUATIONS OF THEIR CYTOTOXIC ACTIVITIES

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(Received August 18, 2022; Revised September 30, 2022; Accepted October 3, 2022)

ABSTRACT. A series of new thiophene derivatives was prepared through nucleophilic substitution reactions of the precursor *N*-(4-substituted-phenyl)-5-(2-chloroacetamido)-4-cyano-3-methylthiophene-2-carboxamides **4a** and **4b** with different sulfur and/or nitrogen nucleophilic reagents (namely; mercaptoacetic acid**,** 2-mercaptobenzothiazole, 5-(phenylamino)-1,3,4-thiadiazole-2-thiol, 2-mercapto-4,6-dimethylnicotinonitrile**,** 3-arylazo-4-mercapto-4-(phenylamino)-but-3-en-one derivatives, ammonium thiocyanate, piperidine and/or morpholine). The structures of the prepared thiophene compounds were characterized by spectral analysis. Their cytotoxicity was evaluated against two human cancer cell lines (HepG2 and MCF-7) and indicated promising results. Pretreatment of HepG2 cells with the tested compound **4b** sensitized the cells to the cytotoxicity of sorafenib, leading to a significant decrease in the IC_{50} from 3.9 to 0.5 µM.

KEY WORDS: *N*-(Thienyl)-2-chloroacetamide, Bis-thiophene, Thieno[2,3-*d*]pyrimidine, Ammonium thiocyanate, Cytotoxicity

INTRODUCTION

The chemistry of heterocyclic compounds occupies a crucial role in drug discovery and development. Thiophenes are one of the commonly found heterocyclic ring systems in many biologically active compounds that have found significant applications in different disciplines [1]. In addition, various substituted thiophenes have been designed and synthesized as drug-like candidates with different pharmacological actions and therapeutic products, like the best-selling drugs olanzepine [2], tinoridine [3], suprofen [4], ritonavir [5], rosiglitazone [6], brotizolam [7], etizolam [8], tiagabine [9], tioconazole, and sertaconazole [10]. As a result, they display a myriad of medicinal potent including anti-influenza virus [11-13], anti-inflammatory [14, 15], anti-glioma [16], antimicrobial [17, 18], antioxidant [19, 20], antitumor [21], human carcinoma growth inhibitors [22], anti-Hepatitis B. virus [23], solar cell [24, 25], dyeing and corrosion [26] properties. Furthermore, and according to the literature, thiophene-based compounds have applications ranging from anti-cancer chemotherapy to the treatment of a variety of other diseases [27, 28]. Several human tumor cell lines, including cervical cancer, gastric cancer, colorectal cancer, oesophageal cancer, lung cancer, hepatocellular carcinoma, oral cancer, osteosarcoma, prostate cancer, ovarian cancer, and breast carcinoma, have shown inhibitory activity in vitro and in vivo [29-36]. The chloroacetamides possess key structural features, including an easy replacement of chlorine atoms, which make them highly attractive synthetic platforms for synthesizing diverse heterocyclic systems. In the present study, we report on the reactivity of *N*-(4-chloro/acetamidophenyl)-5-(2-chloroacetamido)-4-cyano-3-methylthiophene-2-carboxamides **4a,b** toward various types of sulfur and nitrogen nucleophiles to prepare a series of novel thiophene derivatives holding both pharmacophore and evaluation the cytotoxic activity.

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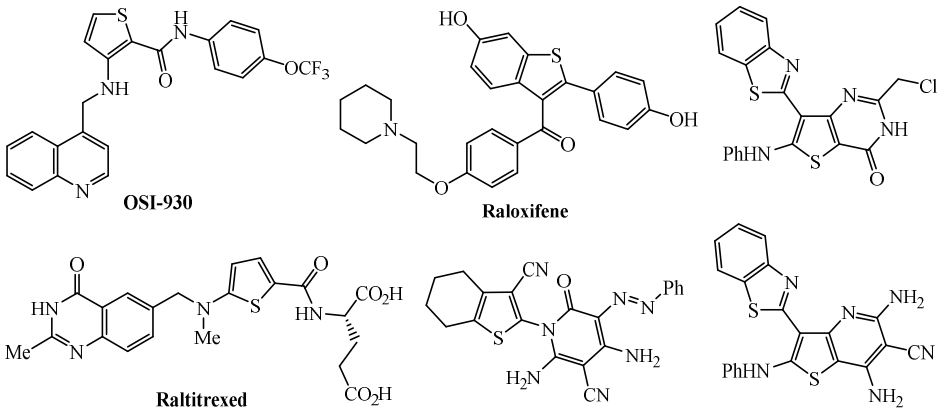
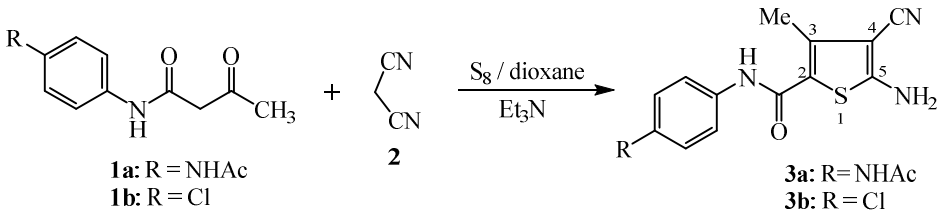


Figure 1. Examples of potent cytotoxic thiophene-based compounds.

RESULTS AND DISCUSSION

The 2-aminothiophene derivatives **3a** and **3b** were easily accessible via the Gewald's reaction between *N*-aryl-3-oxobutanamide derivatives **1**, elemental sulfur, and malononitrile (**2**) according to the method prescribed in literature [37] (Scheme 1). The structures of compounds **3a** and **3b** were secured by their compatible IR, ^{1}H NMR, and ^{13}C NMR analyses. The infrared spectrum of **3a** (as example) displayed absorption bands at 3443, 3302, and 3214 cm^{-1} (NH_2 and N-H), 2207 cm^{-1} (nitrile, -C≡N) and 1660 cm^{-1} (carbonyl, C=O). The ^{1}H NMR signals are identified as singlet at δ 2.00 and 2.34 ppm to indicate the protons of two methyl groups ($NHCOCH_3$ and thiophene-CH_3). The four aromatic protons are resonated as a massive singlet at δ 7.48 ppm. In addition, the two protons of the -NH_2 group resonated as a singlet at δ 7.72 ppm, while the protons of two N-H functions are resonated as singlet signals at δ 9.49 and 9.87 ppm. The ^{13}C NMR spectrum of compound **3a** exhibited signals for the aliphatic carbon atoms in the expected regions, δ 14.91 (CH_3-thiophene), and δ 23.92 ppm ($NHCOCH_3$). Also, the carbon signal at δ 115.52 ppm is related to nitrile carbon (-C≡N). The signals for aromatic carbon atoms were resonated at δ 119.16 (2C), 120.90 (2C), 134.04, and 135.14 ppm. The signals for thiophene ring carbon atoms were observed at δ 87.85, 113.14, 140.10, and 160.23 ppm. In addition, the carbon signals of carbonyl functions were resonated at δ 165.10 and 168.01 ppm.



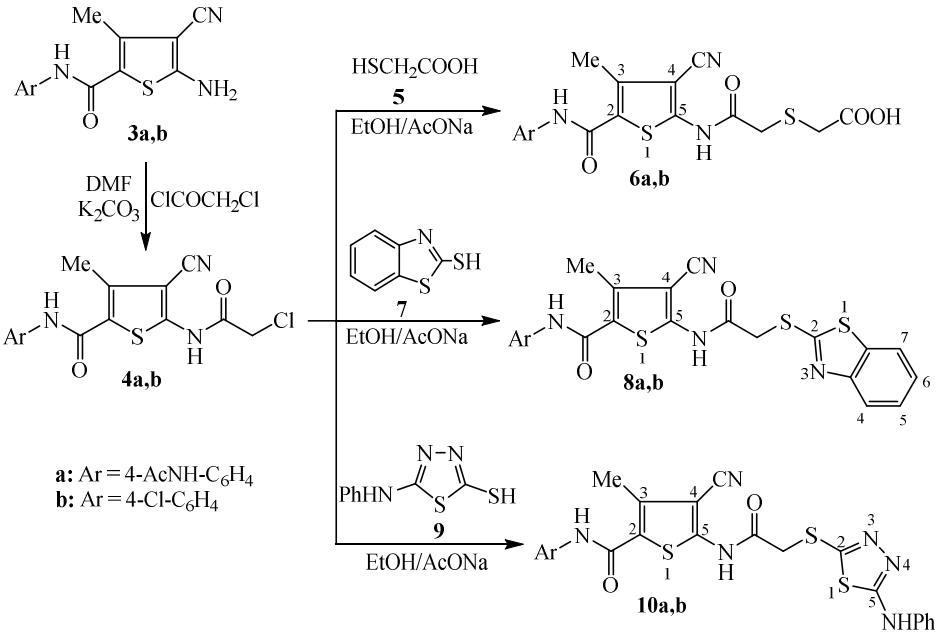
Scheme 1. Synthesis of 2-aminothiophenes **3a** and **3b**.

Chloroacetylation of 2-aminothiophenes **3a** and **3b** was carried out by stirring with chloroacetyl chloride in dimethylformamide and potassium carbonate to furnish the corresponding thienyl 2-chloroacemide derivatives **4** to be utilized as key synthon for the synthesis of polyfunctional substituted heterocycles. The structures of compounds **4a** and **4b** were confirmed by spectroscopic techniques including IR, ^{1}H NMR and ^{13}C NMR. The infrared spectrum of **4b** (as an example) displayed absorption bands at 3277 and 3242 (N-H), 2227 (nitrile, -C≡N), and 1704 and 1640 cm^{-1} (two carbonyl groups). The ^{1}H NMR showed several signals, which were identified as singlet signals at δ 2.46 and 4.53 ppm to indicate the protons of methyl (thiophene-CH_3) and methylene ($COCH_2Cl$) groups. The doublet signals for the aromatic protons are resonated at δ 7.39 and 7.67 ppm. The two protons of N-H groups are resonated as singlet signals at δ 10.22 and 12.42 ppm. The ^{13}C NMR spectrum of compound **4b** exhibited signals for the aliphatic carbon atoms in the expected regions, δ 14.41 (CH_3-thiophene) and 42.35 ppm (CH_2), in addition to the carbon signal of nitrile carbon (-C≡N) at δ 113.78 ppm. The signals of aromatic carbon atoms were indicated by their chemical shifts, δ, 122.05 (2C), 128.62 (2C), 137.63, and 138.50 ppm. The signals for thiophene ring carbon atoms were observed at δ 96.43, 123.06, 149.12 and 160.39 ppm. In addition, the signals of carbonyl groups were resonated at δ 160.51 and 165.72 ppm.

The nucleophilic substitution of the chlorine atom in chloroacetamide derivatives **4** by sulfur-based nucleophiles (namely; 2-mercaptoacetic acid, 2-mercaptobenzothiazole, 5-(phenylamino)-1,3,4-thiadiazol-2-thiol) was investigated (Scheme 2). Thus, the reaction of chloroacetamide derivatives **4** with 2-mercaptoacetic acid was proceeded by heating in ethanol and sodium acetate to afford the corresponding sulfides **6a** and **6b**. The replacement of chlorine atom from chloroacetamide derivatives **4** by 2-mercaptobenzothiazole and/or 5-(phenylamino)-1,3,4-thiadiazol-2-thiol requires heating in ethanol in the presence of sodium acetate to afford the corresponding sulfide derivatives **8a,b** and **10a,b**, respectively. The structures of sulfide-containing compounds **6**, **8**, and **10** were confirmed by spectroscopic means including IR, ^{1}H NMR and ^{13}C NMR. The infrared spectrum of **8b** (as an example) displayed absorption bands at 3264 and 3227 cm^{-1} indicating the imino functions (N-H), 2223 cm^{-1} for the nitrile group in addition to absorptions at 1695 and 1634 cm^{-1} for the carbonyl (C=O) groups. The ^{1}H NMR spectrum showed signals were identified as, two-singlet signals at δ 2.47 and 4.60 ppm corresponding to the protons of methyl group (thiophene-CH_3) and methylene group (S-CH_2) respectively. The aromatic protons are resonating as doublet and triplet of in the downfield region at δ 7.34-8.02 ppm, while the protons of N-H functions were displayed as singlet signals at δ 10.15 and 12.53 ppm. The ^{13}C NMR spectrum of compound **8b** exhibited signals for the aliphatic carbon atoms in the expected regions, δ 14.39 (CH_3-thiophene), and 36.20 ppm (CO-$\underline{CH_2}$-S). Also, the carbon signal at δ 114.09 ppm is related to nitrile carbon (-C≡N). The signals for aromatic carbon atoms were indicated by their chemical shifts, δ 121.19, 122.03 (2C), 122.53, 124.76, 126.57, 128.63 (2C), 135.33, 137.64, 138.65, and 152.43 ppm. The signals for the thiophene ring carbons were observed at δ 96.15, 127.60, 138.73, and 149.25 ppm. The carbon signal of thiazole-C2 is resonated at δ 165.57 ppm. In addition, the carbon signals of carbonyl groups are showed at δ 160.42 and 166.72 ppm (C=O).

Heating of thienyl chloroacetamides **4** with 2-mercapto-4,6-dimethylnicotinonitrile **(11)** in ethanolic solution of sodium ethoxide furnished the corresponding 3-aminothieno[2,3-*b*]pyridine scaffolds **12a** and **12b**. The reaction starts through nucleophilic substitution of the chlorine atom from chloroacetamide **4** followed by intramolecular cyclization between methylene group and the nitrile function (Scheme 3). The IR spectrum of **12b** (as an example) revealed absorption bands at 3344 and 3214 cm^{-1} related to the -NH_2 and N-H functions. The absorption bands at 2232 and 1635 cm^{-1} were assigned to the nitrile (-C≡N) and the carbonyl (C=O) groups, respectively. The ^{1}H NMR spectrum of compound **12b** exhibited three singlet signals in the up field region at δ 2.47, 2.52, and 2.74 ppm, corresponding to the protons of three methyl groups (thiophene-CH_3, pyridine-4-CH_3, pyridine-6-CH_3), respectively. The aromatic protons are exhibited as doublet

signals at δ 7.39 and 7.69 ppm. The protons of N-H functions are resonated down field as two singlet signals at δ 8.60 and 10.19 ppm. The ^{13}C NMR spectrum of compound **12b** displayed, besides the expected aromatic signals, three characteristic signals at δ 14.44, 19.99 and 23.95 ppm assigned to the carbon atoms of methyl groups (thiophene-CH_3, pyridine-4-CH_3 and pyridine-6-CH_3), respectively. In addition, the signals of carbonyl groups were resonated at δ 159.94 and 160.62 ppm (C=O).

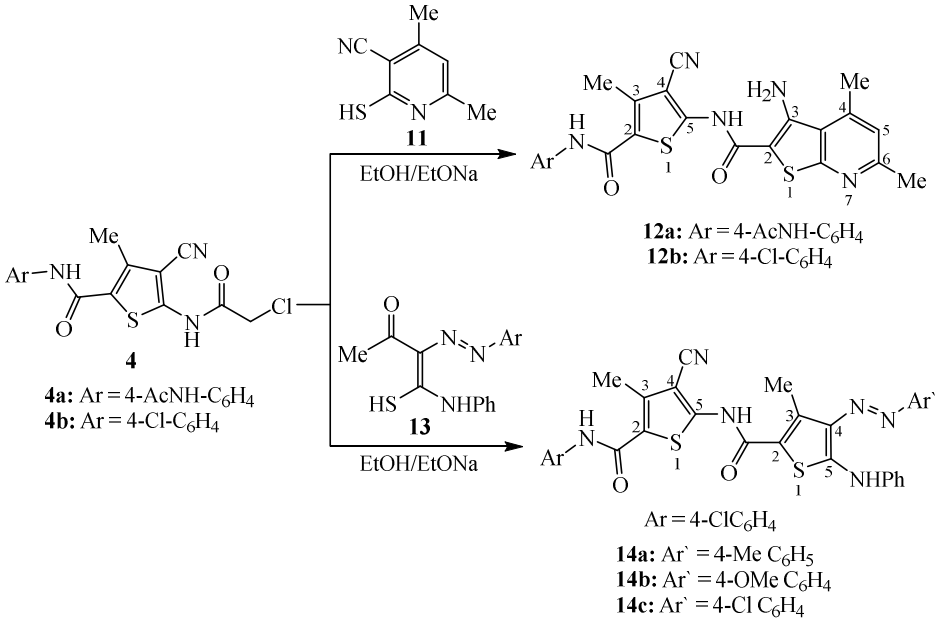


Scheme 2. Synthesis of sulfide derivatives **6**, **8** and **10**.

The heterocyclization reaction of 3-arylazo-4-mercapto-4-(phenylamino)but-3-enone derivatives **13a-c** with chloroacetamide compound **4b** has been achieved by heating in sodium ethoxide-ethanol solution to produce the corresponding *N*-(4-chlorophenyl)-4-cyano-5-(4-arylazo-5-(phenylamino)-thiophene-2-carboxamido)thiophene-2-carboxamide derivatives **14a-c** (Scheme 3). The structure of compounds **14a-c** was confirmed by spectroscopic techniques including IR, ^{1}H NMR and ^{13}C NMR. The infrared spectrum of **14b** (as an example) displayed absorption bands at 3410 and 3251 cm^{-1} indicating the imino functions (N-H), 2210 cm^{-1} for the nitrile group, in addition to absorption at 1638 cm^{-1} assigned to the carbonyl (C=O) groups. The ^{1}H NMR signals were identified as singlet signals at δ 2.46, 2.71 and 3.81 ppm indicated the protons of two methyl groups (thiophene-CH_3) and methoxy group (OCH_3), respectively. The doublet and triplet signals in the region δ 7.05-7.79 ppm identified the aromatic protons. The three protons of N-H functionalities were resonated as singlet signals at δ 10.17, 11.55 and 13.22 ppm.

Heterocyclization of *N*-(thienyl)-2-chloroacetamides **4** upon treatment with ammonium thiocyanate **15** in boiling ethanol did not furnished at the expected product, thienyl thiazolidine-4-one hybrid **17**$_{iii}$ that produced through intramolecular cyclization of the thiocyanate intermediate **16** followed by Dimroth-like rearrangements [38]. Fortunately, *N*-aryl-5-iminothiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine-7-carboxamide derivatives **18a** and **18b** have been isolated as a sole product in each case (Scheme 3). We assume that the course of the reaction proceeds via a

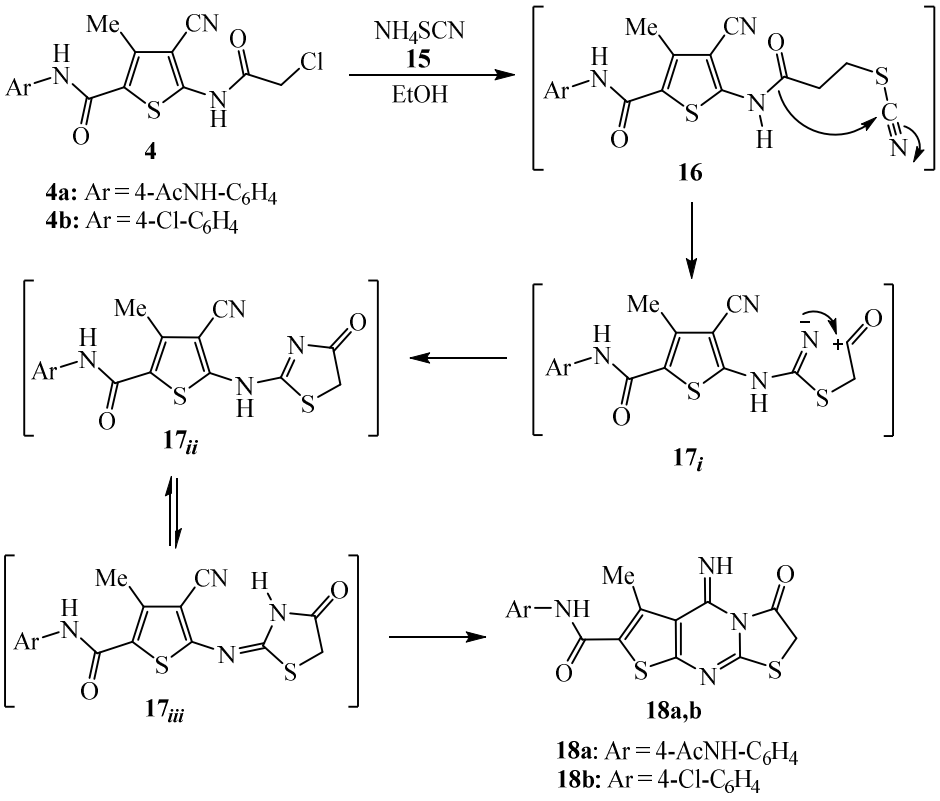
nucleophilic substitution of the chlorine atom from compound **4** followed by a cyclization of the produced thiocyanate **16** to 2-(thienylamino)thiazol-4(5*H*)-ones $\mathbf{17_{ii}}$ and then their tautomeric forms $\mathbf{17_{iii}}$ [39]. Surprisingly, a ring closure of 2-(thienylimino)thiazolidin-4-one $\mathbf{17_{iii}}$ through nucleophilic addition of imino group (=NH-) on the nitrile group furnished the thiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine ring systems. The structure of compound **18** was confirmed by spectroscopic tools including IR, ^{1}H NMR and ^{13}C NMR. The infrared spectrum of **18b** (as an example) did not show any absorption related to the nitrile group and displayed absorptions at 3344 and 3201 cm^{-1} to announce the presence of imino (N-H) groups. Two absorption bands were observed at 1698 and 1662 cm^{-1} were attributed to the carbonyl (C=O) functional groups. The ^{1}H NMR spectrum displayed signals were identified as singlet signals at δ 2.66 and 3.96 ppm to identify the protons of one methyl group and two protons of methylene group (thiazole-CH_2), respectively. The doublet signals at δ 7.40 and 7.69 ppm indicated the four aromatic protons. The two protons of N-H functions are resonated as singlet signals at δ 10.42 and 12.63 ppm. The ^{13}C NMR spectrum of compound **18b** exhibited signals for the aliphatic carbon atoms in the expected regions, δ 16.10 (CH_3-thiophene), and 32.91 ppm (thiazole-CH_2). The signals for aromatic carbon atoms were indicated by their chemical shifts: δ 121.81 (2C), 128.69 (2C), 133.44, and 137.61 ppm. The carbon signals of thiophene ring were observed at δ 113.10, 124.63, 127.67, and 159.00 ppm. The signals of pyrimidine ring carbon were showed at δ 166.36 (C=NH), 166.74 (C=N) ppm. In addition, the signals of carbonyl groups were resonated at δ 161.24 (NH-C=O) and 170.39 ppm (thiazole-C=O).



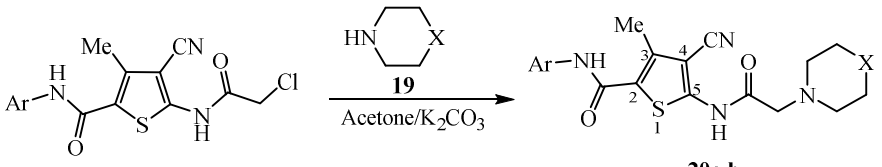
Scheme 3. Synthesis of thiophene derivatives **12a,b** and **14a-c**.

Finally, the nucleophilic substitution of the chlorine atom in thienyl 2-chloroacetamide derivatives **4** by nitrogen-based nucleophiles (namely; morpholine and/or piperidine) was carried out in acetone and anhydrous K_2CO_3 to afford *N*-(4-chlorophenyl)-4-cyano-3-methyl-5-(2-morpholino/piperidino)acetamido)thiophene-2-carboxamides **20a** and **20b** (Scheme 5). The structures of compounds **20a** and **20b** were confirmed by spectroscopic techniques including IR,

^{1}H NMR, and ^{13}C NMR. The infrared spectrum of **20b** (as an example) demonstrated absorption bands at 3361 and 3242 cm^{-1} indicating the imino functions (N-H) and at 2211 cm^{-1} for the nitrile group. Two absorption bands were observed at 1703 and 1654 cm^{-1} are attributed to the carbonyl (C=O) groups. The ^{1}H NMR signals were identified as singlet signals at δ 1.48 (-N-$CH_2CH_2\underline{CH_2}$-), 1.70 (-N-CH_2-$\underline{CH_2}$-CH_2-), and 3.03 (-N-$\underline{CH_2}$-CH_2-CH_2-) ppm to indicate the ten protons of piperidine ring. The three protons of a methyl group (thiophene-CH_3) and the two diastereotopic protons of a methylene group (CO-CH_2) were observed at δ 2.41 and 3.73 ppm, respectively. The doublet signals at δ 7.33 and 7.67 ppm indicated the four aromatic protons. The proton of N-H function is resonating as a singlet at δ 9.81 ppm. The ^{13}C NMR spectrum of compound **20b** exhibited signals for the aliphatic carbon atoms in the expected regions, δ 14.53, 21.85, 23.17, 53.17 and 59.95 ppm assigned to carbon atoms of CH_3, CH_2, and piperidine ring. The carbon signal that recorded at δ 116.46 ppm is related to the nitrile carbon (-C≡N). The signals of aromatic carbon atoms were indicated by their chemical shifts, 121.75 (2C), 126.88, 128.46 (2C), and 138.22 ppm. The carbon signals for thiophene ring were observed at δ 96.51, 118.50, 121.79, and 138.56 ppm. In addition, the carbon signals of carbonyl groups were resonated at δ 161.55 and 167.96 ppm (C=O).



Scheme 4. Synthesis of thiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine derivatives **18a** and **18b**.



4b: Ar = 4-Cl-C_6H_4

20a,b

20a: X= O, Ar = 4-Cl-C_6H_4
20b: X= CH, Ar = 4-Cl-C_6H_4

Scheme 5. The reaction of thienyl chloroacetamide derivative **4b** with morpholine and/or piperidine

In vitro cytotoxic activity

Newly synthesized thiophene compounds **4**, **6**, **8**, **10**, **12**, **14**, **17**, and **20** were evaluated for their effects on the viability of two human cancer cell lines: HepG2 (hepatocellular carcinoma), and MCF-7 (mammary gland breast cancer). Our results indicated that most of the synthesized thiophene derivatives exhibited moderate cytotoxic effects toward the two tested human cancer cell lines (Table 1). Compounds **4a, 4b, 14a,** and **14b** exhibited the highest cytotoxic effect against the tested cell lines HepG2 and MCF-7, their IC_{50} values were very close to the standard drug Sorafenib. In addition, the other tested compounds **6**, **8**, **10**, **12**, **17**, and **20** exhibited moderate and weak cytotoxic effects.

Table 1. Cytotoxic activity of the synthesized thiophene compounds against tumor cells.

Compound Number	IC_{50} (μM)	
	HepG2	MCF-7
4a	66±1.20	50±0.47
4b	54±0.25	50±0.53
6b	100±2.00	100±1.10
8b	73±1.50	82±0.58
10a	98±0.14	100±0.47
10b	82±1.75	100±0.98
12a	98±1.65	100±1.00
12b	79±1.24	95±1.40
14a	57±0.13	72±0.86
14b	58±0.87	65±0.45
14c	62±0.24	69±0.36
17a	94±1.87	96±1.50
17b	93±2.30	86±2.40
20a	73±0.56	81±2.10
20b	72±0.61	81±1.90

The analysis revealed that compound **4a**, **4b**, **14a** and **14b** have cytotoxic activity at the tested 50 μM concentration after 48 hours. The potential activity as a chemosensitizer agent for the four compounds was tested against HepG2. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of hepatocellular carcinoma cell line (HepG2) (Figure 2 and Figure 3). The results of statistical analysis of drug (*in vitro* cytotoxicity) revealed a significant difference between sorafenib alone and each of **4a**, **4b**, **14a**, and **14b** combined with sorafenib (Figure 4).

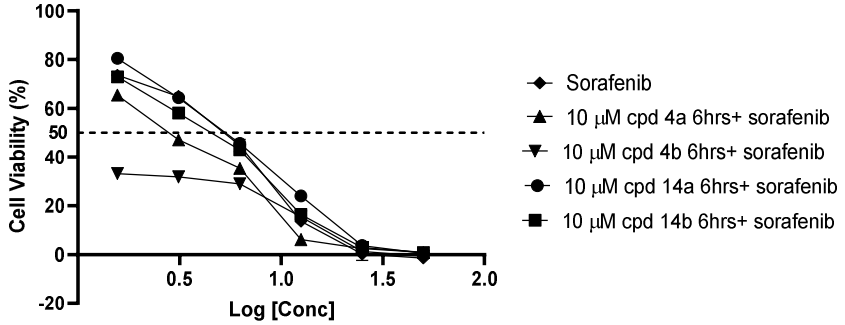


Figure 2. Pretreatment of HepG2 cells with the 10 μM of the tested compounds for 6 hours sensitized the cells to the cytotoxicity of sorafenib leading to a significant decrease in the concentration that is required for killing 50% of cells.

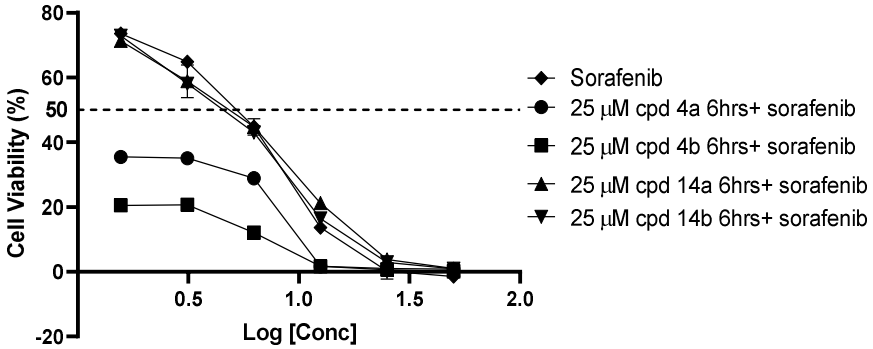


Figure 3. Pretreatment of HepG2 cells with the 25 μM of the tested compounds for 6 hours sensitized the cells to the cytotoxicity of sorafenib leading to a significant decrease in the concentration that is required for killing 50% of cells.

Table 2. Cytotoxic activity of the synthesized thiophene compounds **4a**, **4b**, **14a** and **14b** treated with Sorafenib against human tumor cell HepG2.

Treatment	IC_{50} (μM)
10 μM compound **4a** 6 hrs + **sorafenib**	2.6±0.024
10 μM compound **4b** 6 hrs + **sorafenib**	1.2±0.06
10 μM compound **14a** 6 hrs + **sorafenib**	4.7±0.18
10 μM compound **14b** 6 hrs + **sorafenib**	3.7±0.02
25 μM compound **4a** 6 hrs + **sorafenib**	1.2±0.04
25 μM compound **4b** 6 hrs + **sorafenib**	0.5±0.004
25 μM compound **14a** 6 hrs + **sorafenib**	4.0±0.019
25 μM compound **14b** 6 hrs + **sorafenib**	3.8±0.038
Sorafenib	3.9±0.11

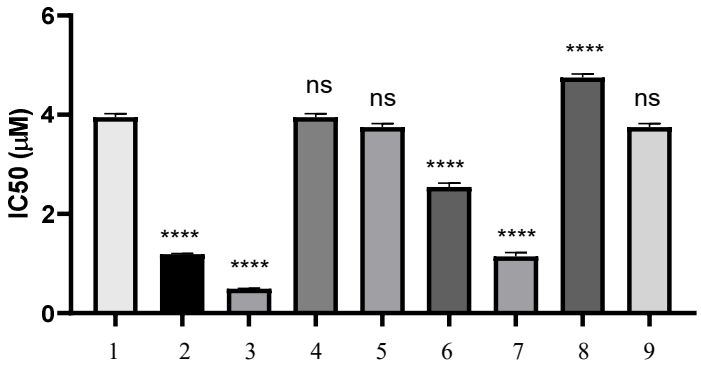


Figure 4. A significant chemosensitizing activity for compounds **4a** and **4b** at the both tested concentrations 10 and 25 μM ($p<0.0001$, ****) while compound **14a** and **14b** were nonsignificant ($p\geq0.05$, ns). (1 = Sorafenib, 2 = 25 μM cpd **4a** 6 hrs + soraferib, 3 = 25 μM cpd **4b** 6 hrs + soraferib, 4 = 25 μM cpd **14a** 6 hrs + soraferib, 5 = 25 μM cpd **14b** 6 hrs + soraferib, 6 = 10 μM cpd **4a** 6 hrs + soraferib, 7 = 10 μM cpd **4b** 6 hrs + soraferib, 8 = 10 μM cpd **14a** 6 hrs + soraferib, and 9 = 10 μM cpd **14b** 6 hrs + soraferib).

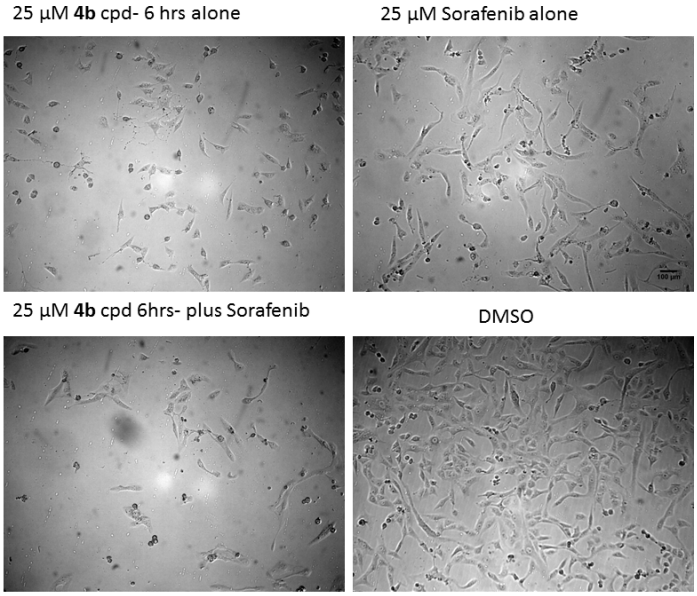


Figure 5. Cytotoxic activity of the synthesized thiophene compound 4b against HepG2 either alone or in combination with sorafenib.

Upon treatment of HepG2 cells with various 25 μM of compound **4b** for 6h, appreciable changes in the cell morphology were evident. HepG2 cells were smaller, distorted, shrank, condensed nuclei and round dead cells in comparison to the DMSO-treated cells (Figure 5).

EXPERIMENTAL

Melting points were determined on a Gallenkamp electric instrument. IR spectra (KBr discs) were recorded on Thermo Scientific Nicolet iS10 FTIR spectrometer. The ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a JEOL spectrometer (Nuclear Magnetic Resonance Unit, Faculty of Science, Mansoura University, Egypt) using $CDCl_3$ or DMSO-d_6 as solvents. The elemental (C, H and N) analyses were determined on Perkin-Elmer 2400 analyzer (Microanalytical Unit, Cairo University, Egypt).

*Synthesis of 5-amino-N-aryl-4-cyano-3-methylthiophene-2-carboxamides **3a** and **3b***

A mixture of *N*-aryl-3-oxobutanamide derivative **1** (0.02 mol), elemental sulfur (0.64 g, 20 mmol) and malononitrile (1.32 g, 20 mmol) in 30 mL ethyl alcohol and 0.2 mL triethylamine. The reaction was preceded by heating under reflux for 3 hours. The precipitate that formed on cooling to 25 ºC was filtered. Recrystallization of the crude products was achieved by heating in ethyl alcohol to separate the 2-aminothiophene derivatives **3a** and **3b**.

*N-(4-Acetamidophenyl)-5-amino-4-cyano-3-methylthiophene-2-carboxamide (**3a**).* Gray crystals, yield 47%, m.p. 284-286 °C. IR (ν/cm^{-1}): 3443, 3302, 3214 (NH_2 and N-H), 2207 (C≡N), broad at 1660 (C=O). ^{1}H NMR (δ/ppm): 2.00 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 7.48 (s, 4H, Ar-H), 7.72 (s, 2H, NH_2), 9.49 (s, 1H, NH), 9.87 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.91, 23.92, 87.85, 113.14, 115.52, 119.16 (2C), 120.90 (2C), 134.04, 135.14, 140.10, 160.23, 165.10, 168.01. Anal. calcd. for $C_{15}H_{14}N_4O_2S$ (314.08): C, 57.31; H, 4.49; N, 17.82%. Found: C, 57.52; H, 4.53; N, 17.70%.

*5-Amino-N-(4-chlorophenyl)-4-cyano-3-methylthiophene-2-carboxamide (**3b**).* Brown crystals, yield 57%, m.p. 200-202 °C; lit. m.p. 199-201 °C [37]. IR (ν/cm^{-1}): 3371, 3307, 3198 (NH_2 and N–H), 2203 (C≡N), 1643 (C=O). ^{1}H NMR (δ/ppm): 2.35 (s, 3H, CH_3), 7.34 (d, J = 9.00 Hz, 2H, Ar-H), 7.61 (d, J = 9.00 Hz, 2H, Ar-H), 7.77 (s, 2H, NH_2), 9.68 (s, 1H, NH). Anal. calcd. for $C_{13}H_{10}ClN_3OS$ (291.75): C, 53.52; H, 3.45; N, 14.40%. Found: C, 53.44; H, 3.52; N, 14.46%.

*Synthesis of N-4-aryl-5-(2-chloroacetamido)-4-cyano-3-methylthiophene-2-carboxamides **4a** and **4b***

To a solution of 2-aminothiophene derivative **3a** or **3b** (5 mmol) in 20 mL DMF and anhydrous K_2CO_3, chloroacetyl chloride (0.56 mL, 7 mmol) was added. The reaction mixture was stirred for 6 hours. The mixture was poured into ice-cold water. The obtained precipitate were collected, filtered, dried and recrystallized by heating in EtOH: DMF mixture (4:1) to give the corresponding thienyl chloroacetamide derivatives **4a** and **4b**, respectively.

*N-(4-Acetamidophenyl)-5-(2-chloroacetamido)-4-cyano-3-methylthiophene-2-carboxamide (**4a**).* Dark gray crystals, yield 76%, m.p. 298-300 °C. IR (ν/cm^{-1}): 3296, 3234 (N–H), 2225 (C≡N), 1708, 1664 (C=O). ^{1}H NMR (δ/ppm): 2.01 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 4.53 (s, 2H, CH_2), 7.50-7.55 (m, 4H, Ar-H), 9.92 (s, 1H, NH), 10.03 (s, 1H, NH), 12.39 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.32 (CH_3), 23.94 (CH_3), 42.32 (CH_2), 96.43, 113.69, 119.19 (2C), 121.00 (2C), 123.58, 133.72, 135.53, 137.76, 148.75, 160.04, 165.60, 168.07. Anal. calcd. for $C_{17}H_{15}ClN_4O_3S$ (390.06): C, 52.24; H, 3.87; N, 14.34%. Found: C, 52.46; H, 3.94; N, 14.26%.

N-(4-Chlorophenyl)-5-(2-chloroacetamido)-4-cyano-3-methylthiophene-2-carboxamide (4b). Brown crystals, yield 65%, m.p. 262-264 °C. IR (ν/cm^{-1}): 3277, 3242 (N–H), 2227 (C≡N), 1704, 1640 (C=O). ^{1}H NMR (δ/ppm): 2.46 (s, 3H, CH_3), 4.53 (s, 2H, CH_2), 7.39 (d, *J* = 8.50 Hz, 2H, Ar-H), 7.67 (d, *J* = 8.50 Hz, 2H, Ar-H), 10.22 (s, 1H, NH), 12.42 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.41 (CH_3), 42.35 (CH_2), 96.43, 113.78, 122.05 (2C), 123.06, 128.62 (2C), 137.63, 138.50, 149.12, 160.39, 160.51, 165.72. Anal. calcd. for $C_{15}H_{11}Cl_2N_3O_2S$ (366.23): C, 48.93; H, 3.01; N, 11.41%. Found: C, 48.76; H, 3.10; N, 11.31%.

General procedure for the synthesis of sulfide derivatives 6, 8 and 10

To a suspension of thienyl chloroacetamide derivative **4a** or **4b** (2 mmol) and 0.50 g sodium acetate in 30 mL ethanol, thioglycolic acid (**5**) (0.18 mL, 2 mmol), 2-mercaptobenzothiazole (**7**) (0.33 g, 2 mmol) and/or 5-(phenylamino)-1,3,4-thiadiazole-2-thiol (**9**) (0.42 g, 2 mmol) was added. The mixture was heated under reflux for 3-4 hour and then poured into ice–H_2O. The precipitate was filtered, washed with cold ethanol and then recrystallized from ethanol to produce the corresponding sulfide derivatives **6**, **8** and **10**, respectively.

*2-((2-((5-((4-Acetamidophenyl)carbamoyl)-3-cyano-4-methylthiophen-2-yl)amino)-2-oxoethyl)-thio)acetic acid (**6a**)*. Brown crystals, yield 76%, m.p. 194-196 °C. IR (ν/cm^{-1}): 3348, 3268 (N–H), 2221 (C≡N), 1674, 1644 (C=O). ^{1}H NMR (δ/ppm): 2.07 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 3.40 (s, 2H, CH_2), 3.65 (s, 2H, CH_2), 7.46 (d, *J* = 8.50 Hz, 2H, Ar-H), 7.52 (d, *J* = 8.50 Hz, 2H, Ar-H), 9.88 (s, 1H, NH), 10.21 (s, 1H, NH), 10.21 (s, 1H, NH). Anal. calcd. for $C_{19}H_{18}N_4O_5S_2$ (446.07): C, 51.11; H, 4.06; N, 12.55%. Found: C, 51.23; H, 4.14; N, 12.50%.

*2-((2-((5-((4-Chlorophenyl)carbamoyl)-3-cyano-4-methylthiophen-2-yl)amino)-2-oxoethyl)-thio)acetic acid (**6b**).* Brown crystals, yield 53%, m.p. 216-218 °C. IR (ν/cm^{-1}): broad at 3262 (N–H), 2222 (C≡N), 1689, 1660 (C=O). ^{1}H NMR (δ/ppm): 2.42 (s, 3H, CH_3), 3.37 (s, 2H, CH_2), 3.71 (s, 2H, CH_2), 7.33 (d, *J* = 9.00 Hz, 2H, Ar-H), 7.62 (d, *J* = 9.00 Hz, 2H, Ar-H), 10.09 (s, 1H, NH), 12.12 (s, 1H, NH). Anal. calcd. for $C_{17}H_{14}ClN_3O_4S_2$ (423.01): C, 48.17; H, 3.33; N, 9.91%. Found: C, 48.26; H, 3.42; N, 9.83%.

*N-(4-Acetamidophenyl)-5-(2-(benzothiazol-2-ylthio)acetamido)-4-cyano-3-methylthiophene-2-carboxamide (**8a**).* Pale yellow crystals, yield 40%, m.p. 256-258 °C. IR (ν/cm^{-1}): 3448 (N–H), 2204 (C≡N), 1660, 1631 (C=O). ^{1}H NMR (δ/ppm): 2.05 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 4.58 (s, 2H, CH_2), 7.43 (t, *J* = 8.00 Hz, 1H, Ar-H), 7.48-7.52 (m, 4H, Ar-H),7.72 (d, *J* = 8.00 Hz, 1H, Ar-H), 7.73 (d, *J* = 8.00 Hz, 1H, Ar-H), 7.79 (d, *J* = 8.50 Hz, 1H, Ar-H), 9.57 (s, 1H, NH), 10.07 (s, 1H, NH), 11.18 (s, 1H, NH). Anal. calcd. for $C_{24}H_{19}N_5O_3S_3$ (521.07): C, 55.26; H, 3.67; N, 13.43%. Found: C, 55.38; H, 3.74; N, 13.32%.

*5-(2-(Benzothiazol-2-ylthio)acetamido)-N-(4-chlorophenyl)-4-cyano-3-methylthiophene-2-carboxamide (**8b**).* Pale brown crystals, yield 42%, m.p. 238-240 °C. IR (ν/cm^{-1}): 3264, 3227 (N–H), 2223 (C≡N), 1695, 1634 (C=O). ^{1}H NMR (δ/ppm): 2.47 (s, 3H, CH_3), 4.60 (s, 2H, CH_2), 7.34-7.37 (m, 4H, Ar-H), 7.45 (t, *J* = 7.50 Hz, 1H, Ar-H), 7.65 (d, *J* = 9.00 Hz, 1H, Ar-H), 7.81 (d, *J* = 8.00 Hz, 1H, Ar-H), 8.02 (d, *J* = 7.50 Hz, 1H, Ar-H), 10.15 (s, 1H, NH), 12.53 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.39 (CH_3), 36.20 (CH_2), 96.15, 114.09, 121.19, 122.03 (2C), 122.53, 124.76, 126.57, 127.60, 128.63 (2C), 135.33, 137.64, 138.65, 138.73, 149.25, 152.43, 160.42, 165.57, 166.72. Anal. calcd. for $C_{22}H_{15}ClN_4O_2S_3$ (498.00): C, 52.95; H, 3.03; N, 11.23%. Found: C, 52.82; H, 3.12; N, 11.16%.

*N-(4-Acetamidophenyl)-4-cyano-3-methyl-5-(2-(5-(phenylamino)-1,3,4-thiadiazol-2-ylthio)-acet-amido)thiophene-2-carboxamide (**10a**).* Pale yellow crystals, yield 41%, m.p. 256-258 °C. IR

(ν/cm^{-1}): broad at 3278 (N–H), 2218 (C≡N), broad at 1654 (C=O). ^{1}H NMR (δ/ppm): 2.00 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 4.34 (s, 2H, CH_2), 6.98 (t, *J* = 7.50 Hz, 1H, Ar-H), 7.32 (t, *J* = 7.50 Hz, 2H, Ar-H), 7.47-7.54 (m, 6H, Ar-H), 9.91 (s, 1H, NH), 10.00 (s, 1H, NH), 10.40 (s, 1H, NH), 12.36 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.31 (CH_3), 23.94 (CH_3), 36.84 (CH_2), 96.03, 113.80, 117.40 (2C), 119.22 (2C), 120.98 (2C), 122.16, 123.21, 129.19 (2C), 133.76, 135.48, 137.85, 140.30, 149.00, 151.57, 160.12, 165.29, 166.90, 168.16. Anal. calcd. for $C_{25}H_{21}N_7O_3S_3$ (563.09): C, 53.27; H, 3.76; N, 17.39%. Found: C, 53.19; H, 3.79; N, 17.31%.

*N-(4-Chlorophenyl)-4-cyano-3-methyl-5-(2-(5-(phenylamino)-1,3,4-thiadiazol-2-ylthio)-acetamido)thiophene-2-carboxamide (**10b**).* Pale brown crystals, yield 46%, m.p. 244-246 °C. IR (ν/cm^{-1}): broad at 3449 (N–H), 2220 (C≡N), 1696, 1635 (C=O). ^{1}H NMR (δ/ppm): 2.46 (s, 3H, CH_3), 4.35 (s, 2H, CH_2), 6.98 (t, *J* = 7.50 Hz, 1H, Ar-H), 7.31 (t, *J* = 7.50 Hz, 2H, Ar-H), 7.38 (d, *J* = 9.00 Hz, 2H, Ar-H), 7.54 (d, *J* = 8.00 Hz, 2H, Ar-H), 7.66 (d, *J* = 9.00 Hz, 2H, Ar-H), 10.19 (s, 1H, NH), 10.40 (s, 1H, NH), 12.40 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.35 (CH_3), 36.82 (CH_2), 96.10, 113.72, 117.44 (2C), 122.00 (2C), 122.13, 122.63, 127.55, 128.59 (2C), 129.17 (2C), 137.63, 138.59, 140.31, 149.19, 151.54, 160.40, 165.25, 166.97. Anal. calcd. for $C_{23}H_{17}ClN_6O_2S_3$ (540.03): C, 51.06; H, 3.17; N, 15.53%. Found: C, 51.18; H, 3.11; N, 15.64%.

*General procedure for the synthesis of N-(3-cyano-5-(4-substituted-phenylcarbamoyl)-4-methylthiophen-2-yl)-3-amino(methyl)thiophenes **12a,b** and **14a-c***

Each of the thienyl chloroacetamide derivative **4a** or **4b** (5 mmol) was stirred for 10 min in sodium ethoxide solution (previously prepared by dissolving 0.23 g sodium granules in 30-mL absolute ethanol). Then the thiol derivatives (5 mmol) (namely; 2-mercapto-4,6-dimethylnicotinonitrile (**11**) or 2-(arylhydrazono)-2-acetyl-thioacetanilide derivatives **13** was added. The mixture was heated on a steam bath for 3-4 hours and then poured into ice water. The solid that formed after neutralization by dilute HCl was filtered and recrystallized from EtOH/DMF mixture (2:1) to afford the bis-thiophene products **12** and **14**, respectively.

*N-(5-((4-Acetamidophenyl)carbamoyl)-3-cyano-4-methylthiophen-2-yl)-3-amino-4,6-dimethyl-thieno[2,3-b]pyridine-2-carboxamide (**12a**).* Yellowish brown crystals, yield 54%, m.p. > 300 °C. IR (ν/cm^{-1}): 3332, 3240 (NH_2 and N–H), 2236 (C≡N), 1666, 1633 (C=O). ^{1}H NMR (δ/ppm): 2.00 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.72 (s, 3H, CH_3), 7.01 (s, 1H, pyridine-H), 7.49 (d, *J* = 8.50 Hz, 2H, Ar-H), 7.55 (d, *J* = 8.50 Hz, 2H, Ar-H), 9.78 (s, 1H, NH), 9.88 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.47 (CH_3-thiophene), 19.88 (CH_3-pyridine), 23.88 (CH_3-pyridine), 96.28, 113.72, 119.24 (2C), 120.78 (2C), 121.76, 123.51, 124.35, 127.29, 129.57, 131.15, 134.27, 135.14, 137.95, 139.44, 141.21, 144.86, 157.79, 159.76, 160.92, 168.08. Anal. calcd. for $C_{25}H_{22}N_6O_3S_2$ (518.12): C, 57.90; H, 4.28; N, 16.21%. Found: C, 57.81; H, 4.22; N, 16.15%.

*3-Amino-N-(5-((4-chlorophenyl)carbamoyl)-3-cyano-4-methylthiophen-2-yl)-4,6-dimethyl-thieno[2,3-b]pyridine-2-carboxamide (**12b**).* Brown crystals, yield 70%, m.p. 284-286 °C. IR (ν/cm^{-1}): 3344, 3214 (NH_2 and N-H), 2232 (C≡N), 1635 broad at (C=O). ^{1}H NMR (δ/ppm): 2.47 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.74 (s, 3H, CH_3), 7.09 (s, 1H, pyridine-H), 7.39 (d, *J* = 9.00 Hz, 2H, Ar-H), 7.69 (d, *J* = 8.50 Hz, 2H, Ar-H), 8.60 (s, 1H, NH), 10.19 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.44 (CH_3-thiophene), 19.99 (CH_3-pyridine), 23.95 (CH_3-pyridine), 97.97, 114.22, 121.84, 121.92 (4C), 122.19 (2C), 127.46, 128.50 (4C), 128.60, 137.75, 138.43, 145.43, 159.94, 160.62. Anal. calcd. for $C_{23}H_{18}ClN_5O_2S_2$ (495.06): C, 55.70; H, 3.66; N, 14.12%. Found: C, 55.58; H, 3.56; N, 14.18%.

*N-(4-Chlorophenyl)-4-cyano-3-methyl-5-(3-methyl-5-(phenylamino)-4-(p-tolylazo)thiophene-2-carboxamido)thiophene-2-carboxamide (**14a**).* Red crystals, yield 38%, m.p. 286-288 °C. IR

(ν/cm^{-1}): 3401 (N–H), 2215 (C≡N), broad at 1643 (C=O). ^{1}H NMR (δ/ppm): 2.32 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 2.62 (s, 3H, CH_3), 7.27 (d, *J* = 7.50 Hz, 4H, Ar-H), 7.35-7.42 (m, 4H, Ar-H), 7.51 (t, *J* = 7.50 Hz, 1H, Ar-H), 7.59 (d, *J* = 7.00 Hz, 2H, Ar-H), 7.69 (d, *J* = 9.00 Hz, 2H, Ar-H), 10.14 (s, 1H, NH), 11.65 (s, 1H, NH), 13.87 (s, 1H, NH). Anal. calcd. for $C_{32}H_{25}ClN_6O_2S_2$ (624.12): C, 61.48; H, 4.03; N, 13.44%. Found: C, 61.53; H, 4.07; N, 13.39%.

*N-(4-Chlorophenyl)-4-cyano-5-(4-((p-anisylazo)-3-methyl-5-(phenylamino)thiophene-2-carboxamido)-3-methylthiophene-2-carboxamide (**14b**).* Dark red crystals, yield 45%, m.p. 296-298 °C. IR (ν/cm^{-1}): 3410, 3251 (N–H), 2210 (C≡N), broad at 1638 (C=O). ^{1}H NMR (δ/ppm): 2.46 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 7.05 (d, *J* = 8.50 Hz, 2H, Ar-H), 7.26 (t, *J* = 7.00 Hz, 1H, Ar-H), 7.39 (d, *J* = 9.00 Hz, 2H, Ar-H), 7.46 (d, *J* = 8.00 Hz, 2H, Ar-H), 7.51 (t, *J* = 8.00 Hz, 2H, Ar-H), 7.69 (d, *J* = 9.00 Hz, 2H, Ar-H), 7.79 (d, *J* = 7.00 Hz, 2H, Ar-H), 10.17 (s, 1H, NH), 11.55 (s, 1H, NH), 13.22 (s, 1H, NH). Anal. calcd. for $C_{32}H_{25}ClN_6O_3S_2$ (640.11): C, 59.95; H, 3.93; N, 13.11%. Found: C, 59.83; H, 3.88; N, 13.97%.

*N-(4-Chlorophenyl)-5-(4-((p-chlorophenyl)azo)-3-methyl-5-(phenylamino)thiophene-2-carboxamido)-4-cyano-3-methylthiophene-2-carboxamide (**14c**).* Dark red crystals, yield 41%, m.p. 300 °C. IR (ν/cm^{-1}): 3421, 3380 (N–H), 2210 (C≡N), 1696 (C=O). ^{1}H NMR (δ/ppm): 2.44 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 7.10 (t, *J* = 7.50 Hz, 1H, Ar-H), 7.28 (t, *J* = 7.50 Hz, 2H, Ar-H), 7.35-7.39 (m, 3H, Ar-H), 7.42-7.53 (m, 5H, Ar-H), 7.70 (d, *J* = 7.00 Hz, 2H, Ar-H), 9.99 (s, 1H, NH), 11.77 (s, 1H, NH), 14.07 (s, H, NH). Anal. calcd. for $C_{31}H_{22}Cl_2N_6O_2S_2$ (644.06): C, 57.68; H, 3.44; N, 13.02;%. Found: C, 57.72; H, 3.47; N, 13.11%.

*Synthesis of N-aryl-5-imino-6-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]thieno[2,3-d]pyrimidine-7-carboxamides **18a** and **18b***

A suspension of chloroacetamide derivative **4a** or **4b** (2 mmol) and ammonium thiocyanate (0.30 g, 4 mmol) in 20 mL ethanol was heated under reflux for 4 hours. The obtained precipitate was collected by filtration and then recrystallized by heating in ethyl alcohol to yield the thiazole-thieno-pyrimidine analogues **18a** and **18b**.

*N-(4-Acetamidophenyl)-5-imino-6-methyl-3-oxo-2,3-dihydro-5H-thiazolo[3,2-a]thieno[2,3-d]-pyrimidine-7-carboxamide (**18a**).* Gray crystals, yield 68%, m.p. 300 °C. IR (ν/cm^{-1}): 3414, 3200 (N-H), 1660, 1618 (C=O). ^{1}H NMR (δ/ppm): 2.01 (s, 3H, CH_3), 2.65 (s, 3H, CH_3), 3.77 (s, 1H, CH_2), 7.52-7.57 (m, 4H, Ar-H), 9.98 (s, H, NH), 10.23 (s, H, NH). ^{13}C NMR (δ/ppm): 16.13 (CH_3), 23.99 (CH_3), 34.30 (SCH_2), 113.14, 119.33 (3C), 120.83 (2C), 125.13, 132.85, 133.81, 135.60, 158.97, 160.96, 166.75, 168.24, 169.95. Anal. calcd. for $C_{18}H_{15}N_5O_3S_2$ (413.06): C, 52.29; H, 3.66; N, 16.94%. Found: C, 52.11; H, 3.60; N, 16.83%.

*N-(4-Chlorophenyl)-5-imino-6-methyl-3-oxo-3,5-dihydro-2H-thiazolo[3,2-a]thieno[2,3-d]pyrimidine-7-carboxamide (**18b**).* Greenish brown crystals, yield 56%, m.p. 290-292 °C. IR (ν/cm^{-1}): 3344, 3201 (N–H), 1698, 1662 (C=O). ^{1}H NMR (δ/ppm): 2.66 (s, 3H, CH_3), 3.93 (s, 2H, CH_2), 7.40 (d, *J* = 8.50 Hz, 2H, Ar-H), 7.69 (d, *J* = 8.50 Hz, 2H, Ar-H), 10.42 (s, 1H, NH), 12.63 (s, 1H, NH). ^{13}C NMR (δ/ppm): 16.10 (CH_3), 32.91 (CH_2), 113.10, 121.81 (2C), 124.63, 127.67, 128.69 (2C), 133.44, 137.61, 159.00, 161.24, 166.36, 166.74, 170.39. Anal. calcd. for $C_{16}H_{11}ClN_4O_2S_2$ (390.00): C, 49.17; H, 2.84; N, 14.33%. Found: C, 49.38; H, 2.91; N, 14.21%.

*Synthesis of N-(4-chlorophenyl)-4-cyano-3-methyl-5-(2-morpholino/piperidinyl acetamido)-thiophene-2-carboxamide **20a** and **20b***

To a suspension of chloroacetamide derivative **4b** (0.37 g, 1 mmol) and anhydrous potassium carbonate (0.14 g) in 15 mL of dry acetone, an equimolar amount of corresponding secondary amine (piperidine or morpholine) (1 mmol) was added. The reaction mixture was refluxed for 5

hours. The mixture was poured into ice-cold water. The solid that produced was filtered, dried and recrystallized by heating in ethyl alcohol.

*N-(4-Chlorophenyl)-4-cyano-3-methyl-5-(2-morpholinoacetamido)thiophene-2-carboxamide (**20a**).* Brown crystals, yield 38%, m.p. 194-196 °C. IR (ν/cm^{-1}): 3378 broad (N–H), 2209 (C≡N), 1704, 1650 (C=O). ^{1}H NMR (δ/ppm): 2.45 (s, 3H, CH_3), 2.62 (t, *J* = 4.50 Hz, 4H, $2CH_2$), 3.45 (s, 2H, CH_2), 3.62 (t, *J* = 4.50 Hz, 4H, $2CH_2$), 7.38 (d, *J* = 9.00 Hz, 2H, Ar-H), 7.68 (d, *J* = 9.00 Hz, 2H, Ar-H), 10.14 (s, 1H, NH). ^{13}C NMR (δ/ppm): 14.39 (CH_3), 52.77 (2C, N-CH_2), 59.91 (CH_2), 65.99 (2C, OCH_2), 96.32, 114.08, 121.99 (2C), 127.48, 128.49 (2C), 128.57, 137.71, 138.33, 150.49, 160.57, 168.68. Anal. calcd for $C_{19}H_{19}ClN_4O_3S$ (418.09): C, 54.48; H, 4.57; N, 13.38%. Found: C, 54.38; H, 4.53; N, 13.43%.

*N-(4-Chlorophenyl)-4-cyano-3-methyl-5-(2-(piperidin-1-yl)acetamido)thiophene-2-carboxamide (**20b**).* Pale brown crystals, yield 40%, m.p. 246-248 °C. IR (ν/cm^{-1}): 3361, 3242 (N–H), 2211 (C≡N), 1703, 1654 (C=O). ^{1}H NMR (δ/ppm): 1.48 (s, 2H, CH_2), 1.70 (s, 4H, $2CH_2$), 2.41 (s, 3H, CH_3), 3.03(s, 4H, $2CH_2$), 3.73 (s, 2H, CH_2), 7.33 (d, *J* = 24 Hz, 2H, Ar-H), 7.67 (d, *J* = 10 Hz, 2H, Ar-H), 9.81 (s, H, NH). ^{13}C NMR (δ/ppm): 14.53, 21.85, 23.17 (2C), 53.17 (2C), 59.95, 96.51, 116.46, 118.50, 121.75 (2C), 121.79, 126.88, 128.46 (2C), 138.22, 138.56, 161.55, 167.96. Anal. calcd. for $C_{20}H_{21}ClN_4O_2S$ (416.11): C, 57.62; H, 5.08; N, 13.44%. Found: C, 57.51; H, 5.00; N, 13.52%.

Cell lines and reagents

HepG2 and MCF-7 cell lines were purchased from Nawah Scientific Company, Egypt. Cells were grown in DMEM medium (BioWhittaker™) supplemented with bovine serum albumin (10%, Life Science Group L, UK, Cat No: S-001B-BR) and with 100 IU/mL penicillin/ streptomycin (100 µg/mL) (Lonza, 17-602E). Sorafenib (SML2653) was obtained from Sigma-Aldrich, solubilized in DMSO and kept at –20°C as a stock solution. The tested compounds were prepared in dimethyl sulfoxide (10 mM stock) (DMSO Cat. No. 20385.02, Serva, Heidelberg, Germany) and stored at –20 °C.

The initial screening and cell viability by MTT assay. According to Skehan *et al.*, procedures with minor modifications [40, 41] the cancer cells were seeded in a 96-well plate (100 µL/well). After overnight incubation at 37 ºC and 5% CO_2, the cells were incubated with 50 µM of each tested compound or DMSO (0.5% V/V). After 48 hours of incubation, MTT (3-(4,5-dimethylthiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/mL phosphate buffered saline (PBS)) was added, and the plate was incubated for 4 hours. After that, acidified sodium dodecyl sulfate (SDS) solution (10% SDS containing 0.01N HCl in 1x PBS) was used to solubilize formazan crystals. The absorbance was measured after 14 hours of incubation at $\lambda_{570-630}$ nm by a Biotek plate reader (Gen5™).

Chemosensitization

According to El-Senduny *et al.* [42] HepG2 cells were pretreated either with 10 or 25 µM of compound **4a**, **4b**, **14a**, **14b** or DMSO for 6 hours then treated with a serial dilution of Sorafenib (50, 25, 12.5, 6.25, 3.125, 1.56 µM) for total 48 hours. The unsensitized cells were treated with Sorafenib alone for 48 hours. Cell viability was determined by MTT assay as mentioned above after pretreatment with the tested compound for 6 hours alone and after the sensitization after 48 hours. IC_{50}% of Sorafenib was calculated by using Prism 8.0 Software.

CONCLUSION

The present study has been focused on the synthesis and investigation the cytotoxic activity of novel thiophene derivatives containing biologically active thiophene nucleus. A series of some novel heterocyclic scaffolds containing thiophene moiety was synthesized through the reaction of *N*-(4-substituted-phenyl)-5-(2-chloroacetamido)-4-cyano-3-methylthiophene-2-carboxamide **4a,b** with various types of (sulfur and nitrogen) nucleophiles in presence of different bases to afford the new thiophene scaffolds. The structures of the synthesized compounds were characterized by spectral analyses then assayed in vitro for cytotoxic activity. Some of the newly synthesized compounds **4a,b** and **14a,b** exhibited significant activity (very strong) compared to the control drug, Sorafenib. (i) acetamidophenyl and chlorophenyl substitution at position 5 of thiophene ring are important for cytotoxic activity, (ii) whereas, CH_3 group at position 4 of thiophene ring (**4a,b** and **14a,b**) would be favorable for the mentioned cytotoxic activity; and (iii) bis-thiophene ring **14a,b** and is expected to offer better cytotoxic activity.

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