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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, biological evaluation and in silico study of bis-thiourea derivatives as anticancer, antimalarial and antimicrobial agents

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Abstract Two sets of bis-thioureas including a *para* series (**8–14**) and a *meta* series (**4**, **5**, **15–19**), were synthesized and evaluated for their anticancer, antimalarial and antimicrobial activities. Most of the synthesized bis-thioureas, except for analogs **8–11**, displayed cytotoxicity against MOLT-3 cell line ($IC_{50} = 1.55–32.32\ \mu M$). Derivatives **5**, **14**, **18** and **19** showed a broad spectrum of anticancer activity. Analogs (**4**, **5**, **8**, **13**, **14**, **18** and **19**) exhibited higher inhibitory efficacy in HepG2 cells than the control drug, etoposide. Significantly, bis-trifluoromethyl analog **19** was the promising potent cytotoxic agent ($IC_{50} = 1.50–18.82\ \mu M$) with the best safety index (1.64–20.60). Antimalarial activity results showed that trifluoromethyl derivative **18** was the most potent compound ($IC_{50} = 1.92\ \mu M$, selective index = 6.86). Antimicrobial activity revealed that bis-thioureas **12**, **18** and **19** exhibited selective activity against Gram-positive bacteria and fungi. Promisingly, the bis-trifluoromethyl derivative **19** was the most potent compound in the series and displayed higher potency, against most of the Gram-positive bacteria and fungi, than that of ampicillin, the reference drug. Among the tested strains of microorganisms, compound **19** inhibited the growth of *Staphylococcus epidermidis* ATCC 12228 and *Micrococcus luteus* ATCC 10240 with the lowest MIC of 1.47 μM. The findings demonstrated that trifluoromethyl group plays a crucial role in their biological activities. Furthermore, the molecular docking was performed to reveal possible binding modes of the compounds against target proteins.

Keywords Thiourea · Trifluoromethyl group · Anticancer activity · Antimalarial activity · Antimicrobial activity · Molecular docking

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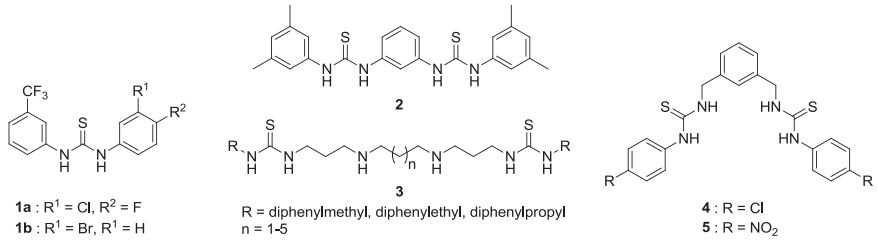
Introduction

Thiourea derivatives have drawn considerable interest in the field of medicine, agriculture and analytical chemistry. These compounds display a diverse range of pharmacological activities including anticancer (Kumar and Chimni 2015; Pingaew et al. 2013; Liu et al. 2015; Vega-Pérez et al.

Fig. 1 Bioactive thioureas



2012; Tahir et al. 2015; Bielenica et al. 2015), antimalarial (Mishra and Batra 2013) and antimicrobial (Vega-Pérez et al. 2012; Tahir et al. 2015; Bielenica et al. 2015; Mishra and Batra 2013; Saeed et al. 2009; Suresha et al. 2011; Stefanska et al. 2015; Cunha et al. 2007) activities. Bioactive thioureas possess specific binding sites known as hydrogen binding area (NH), complementary area (S) and auxiliary binding area (1,3-substituents) (Mishra and Batra 2013). Thiourea has more lipophilicity than urea congener thus rendering its easy penetration to cells. Various structural modifications of thioureas by introducing halogen and trifluoromethyl moieties can improve biological effects of the compounds (Vega-Pérez et al. 2012; Tahir et al. 2015; Bielenica et al. 2015; Suresha et al. 2011; Stefanska et al. 2015). Several studies (Bielenica et al. 2015; Azam et al. 2015; Ehmann and Lahiti 2014; Grillot et al. 2014; Basarab et al. 2013; Charifson et al. 2008) indicated that potent antibacterial activity of halogenated urea/thiourea derivatives such as compound (**1**) (Bielenica et al. 2015) was elucidated by an inhibition of topoisomerase IV (Fig. 1).

Bis-thioureas have been denoted as compounds with two thiourea units which showed significant pharmacological activities. For example, phenyl-bis(phenylthiourea) **2** displayed cytotoxic activity toward multiple cancer cell lines at nanomolar concentrations and its action involved the inhibition of tubulin polymerization (Shing et al. 2014) (Fig. 1). Many alkylated (bis)thiourea polyamine analogs (**3**) showed antitumor activity by acting as a lysine-specific demethylase 1 inhibitor (Sharma et al. 2010; Nowotarski et al. 2015) as well as antimalarial activity against *Plasmodium falciparum* (Verlinden et al. 2015, 2011). In our group, bis-thioureas (**4** and **5**) derived from *m*-xylylenediamine have been revealed to exert potent anticancer activity against HuCCA-1, HepG2 and MOLT-3 cancer cell lines (Pingaew et al. 2013).

In view of the existing reports and continuation of our interest in the search for novel bis-thioureas endowed with potential chemotherapeutic activities, the synthetic bis-thiourea targets have been designed as xylylene-bis(phenylthiourea) (Fig. 2). The core structure of target compounds bears phenylthioureas at *para* and *meta* positions on a xylene ring A, and different substituted groups ($R = CH_3$, OCH_3, Cl, Br, NO_2, CF_3 and $2 \times CF_3$) on phenyl rings B.

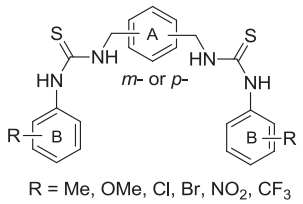


Fig. 2 Designed bis-thiourea derivatives

The importance of our work lies in the possibility that the obtained synthetic bis-thioureas may serve as a useful lead compound for the discovery of novel bioactive agents by using a simple chemical synthetic procedure. Herein, a variety of known bis-thioureas (**4**, **5**, **15** and **19**) and novel bis-thiourea derivatives (**8–14, 16–18**) were synthesized and evaluated for their anticancer, antimalarial and antimicrobial activities. Furthermore, the molecular docking was performed to reveal possible binding modalities of the investigated compounds against target proteins accounting for anticancer (i.e., tubulin) and antimicrobial (i.e., topoisomerase IV) activities. Two-dimensional ligand-protein interaction diagrams also were generated to elucidate crucial binding moieties and interactions.

Materials and methods

Chemistry

Column chromatography was carried out using silica gel 60 (70–230 mesh ASTM). Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} aluminum sheets. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 300 NMR spectrometer (operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C). FTIR spectra were obtained using a universal attenuated total reflectance attached on a Perkin–Elmer Spectrum One spectrometer. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics (microTOF). Melting points were determined using a Griffin melting point apparatus and were uncorrected.

General procedure for the synthesis of bis-thioureas (4, 5, 8–19)

A mixture of xylylenediamine (2 mmol) and appropriate phenylisothiocyanate (4 mmol) in dichloromethane (20 mL) was stirred at room temperature for 3–16 h (monitored by TLC). The solid product formed was filtered, and recrystallized from methanol to give the bis-thiourea derivatives (**4, 5, 8–19**).

^{1}H NMR data of 1,1′-(1,3-phenylenebis(methylene))bis(3-(4-chlorophenyl)thiourea) (**4**) (Pingaew et al. 2013), 1,1′-(1,3-phenylenebis(methylene))bis(3-(4-nitrophenyl)thiourea) (**5**) (Pingaew et al. 2013), 1,1′-(1,3-phenylenebis(methylene))bis(3-(4-methylphenyl)thiourea) (**15**) (Gulgas and Reineke 2008) and 1,1′-(1,3-phenylenebis(methylene))bis(3-(3,5-ditrifluoromethylphenyl)thiourea) (**19**) (Jones et al. 2008) are consistent with that reported in the literature.

*1,1′-(1,4-phenylenebis(methylene))bis(3-(4-methylphenyl) thiourea) (**8**)*

White solid. 80%. Mp 209–210 °C. IR (UATR) cm^{-1}: 3276, 1585, 1540, 1516, 1310, 1189, 956, 788, 662. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 2.25 (s, 6H, 2 × C*H*$_3$), 4.68 (s, 4H, 2 × C*H*$_2$), 7.11 (d, $J = 8.2$ Hz, 4H, Ar*H*), 7.25 (d, $J = 8.2$ Hz, 4H, Ar*H*), 7.27 (s, 4H, Ar*H*), 8.04 (br.s, 2H, N*H*), 9.51 (br.s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 20.9 (2 × CH$_2$), 47.4 (2 × CH$_3$), 124.3 (2 × C-2′, 2 × C-6′), 127.8 (C-2, C-3, C-5, C-6), 129.6 (2 × C-3′, 2 × C-5′), 134.2 (2 × C-4′), 136.9 (2 × C-1′), 138.1 (C-1, C-4), 181.3 (C = S). HRMS-TOF: *m/z* [M + H]$^+$435.1675 (calcd for $C_{24}H_{27}N_4S_2$: 435.1672).

*1,1′-(1,4-phenylenebis(methylene))bis(3-(4-methoxyphenyl) thiourea) (**9**)*

White solid. 79%. mp 209–210 °C. IR (UATR) cm^{-1}: 3245, 1565, 1542, 1509, 1298, 1243, 1032, 970, 831, 727, 679. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 3.72 (s, 6H, 2 × OC*H*$_3$), 4.67 (d, $J = 5.4$ Hz, 4H, 2 × C*H*$_2$), 6.90 (d, $J = 8.8$ Hz, 4H, Ar*H*), 7.23 (d, $J = 8.8$ Hz, 4H, Ar*H*), 7.26 (s, 4H, Ar*H*), 7.91 (br.s, 2H, N*H*), 9.39 (br.s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 47.5 (2 × CH$_2$), 55.7 (2 × OCH$_3$), 114.5 (2 × C-3′, 2 × C-5′), 126.6 (2 × C-2′, 2 × C-6′), 127.7 (C-2, C-3, C-5, C-6), 132.1 (2 × C-1′), 138.2 (C-1, C-4), 157.1 (2 × C-4′), 181.6 (C = S). HRMS-TOF: *m/z* [M + H]$^+$ 467.1581 (calcd for $C_{24}H_{27}N_4O_2S_2$: 467.1570).

*1,1′-(1,4-phenylenebis(methylene))bis(3-(4-chlorophenyl) thiourea) (**10**)*

White solid. 75%. mp 233–234 °C. IR (UATR) cm^{-1}: 3271, 3202, 1590, 1524, 1489, 1332, 1286, 1091, 825, 728. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 4.71 (s, 4H, 2 × C*H*$_2$), 7.31 (s, 4H, Ar*H*), 7.37 (d, $J = 8.7$ Hz, 4H, Ar*H*), 7.48 (d, $J = 8.7$ Hz, 4H, Ar*H*), 8.27 (br.s, 2H, N*H*), 9.69 (br. s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 47.4 (2 × CH$_2$), 125.3 (2 × C-3′, 2 × C-5′), 127.9 (C-2, C-3, C-5, C-6), 128.4 (2 × C-4′), 128.9 (2 × C-2′, 2 × C-6′), 138.0 (C-1, C-4), 138.8 (2 × C-1′), 181.3 (C = S). HRMS-TOF: *m/z* [M + H]$^+$ 475.0581 (calcd for $C_{22}H_{21}Cl_2N_4S_2$: 475.0579).

*1,1′-(1,4-phenylenebis(methylene))bis(3-(4-bromophenyl) thiourea) (**11**)*

White solid. 81%. Mp 208–209 °C. IR (UATR) cm^{-1}: 3271, 3200, 1588, 1537, 1524, 1485, 1334, 1284, 1070, 822. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 4.69 (d, $J = 5.4$ Hz, 4H, 2 × C*H*$_2$), 7.28 (s, 4H, Ar*H*), 7.41 (d, $J = 8.8$ Hz, 4H, Ar*H*), 7.48 (d, $J = 8.8$ Hz, 4H, Ar*H*), 8.24 (br.s, 2H, N*H*), 9.66 (br.s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 47.4 (2 × CH$_2$), 116.6 (2 × C-4′), 125.6 (2 × C-2′, 2 × C-6′), 127.9 (C-2, C-3, C-5, C-6), 131.8 (2 × C-3′, 2 × C-5′), 137.9 (C-1, C-4), 139.2 (2 × C-1′), 181.3 (C = S). HRMS-TOF: *m/z* [M + Na]$^+$ 584.9368 (calcd for $C_{22}H_{20}Br_2N_4NaS_2$: 584.9388).

*1,1′-(1,4-phenylenebis(methylene))bis(3-(4-nitrophenyl) thiourea) (**12**)*

Yellow solid. 76%. mp 254–255 °C. IR (UATR) cm^{-1}: 3310, 1596, 1504, 1328, 1302, 1257, 1112, 850, 698. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 4.75 (s, 4 H, 2 × C*H*$_2$), 7.35 (s, 4H, Ar*H*), 7.86 (d, $J = 8.8$ Hz, 4H, Ar*H*), 8.18 (d, $J = 8.8$ Hz, 4H, Ar*H*), 8.80 (br.s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 47.1 (2 × CH$_2$), 121.1 (2 × C-3′, 2 × C-5′), 124.9 (2 × C-2′, 2 × C-6′), 128.0 (C-2, C-3, C-5, C-6), 137.6 (C-1, C-4), 142.4 (2 × C-4′), 146.9 (2 × C-1′), 180.8 (C = S). HRMS-TOF: *m/z* [M + H]$^+$ 497.1076 (calcd for $C_{22}H_{21}N_6O_4S_2$: 497.1060).

*1,1′-(1,4-phenylenebis(methylene))bis(3-(4-trifluoromethyl-phenyl)thiourea) (**13**)*

White solid. 74%. Mp 215–216 °C. IR (UATR) cm^{-1}: 3230, 1560, 1527, 1515, 1328, 1135, 841, 706. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ (ppm) 4.72 (s, 4H, 2 × C*H*$_2$), 7.32 (s, 4H, Ar*H*), 7.65 (d, $J = 8.6$ Hz, 4H, Ar*H*), 7.73 (d, $J = 8.6$ Hz, 4H, Ar*H*), 8.46 (br.s, 2H, N*H*), 9.94 (br.s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 47.4 (2 × CH$_2$), 122.6 (2 × C-3′, 2 × C-5′), 124.0 (q, $^2J_{CF} = 32$ Hz, 2 × C-4′), 125.2 (q, $^1J_{CF} = 269$ Hz, CF$_3$), 126.1 (2 × C-2′, 2 × C-6′), 128.0 (C-2, C-3, C-5, C-6), 137.8 (C-1, C-4), 143.8 (2 × C-1′), 181.2 (C = S). HRMS-TOF: *m/z* [M + H]$^+$ 543.1122 (calcd for $C_{24}H_{21}F_6N_4S_2$: 543.1106).

*1,1′-(1,4-phenylenebis(methylene))bis(3-(3,5-ditrifluoromethylphenyl)thiourea) (**14**)*

White solid. 70%. Mp 234–235 °C. IR (UATR) cm^{-1}: 3230, 1545, 1382, 1275, 1181, 1140, 955, 893. ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.74 (s, 4H, 2 × CH_2), 7.32 (s, 4H, Ar*H*), 7.72 (s, 2H, Ar*H*), 8.24 (s, 4H, Ar*H*), 8.69 (br. s, 2H, N*H*), 10.00 (br.s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d_6) δ 47.3 (2 × CH_2), 116.7 (2 × C-4′), 122.7 (2 × C-2′, 2 × C-6′), 123.7 (q, $^1J_{CF} = 271$ Hz, CF_3), 128.0 (C-2, C-3, C-5, C-6), 130.6 (q, $^2J_{CF} = 32$ Hz, 2 × C-3′, 2 × C-5′), 137.6 (C-1, C-4), 142.4 (2 × C-1′), 181.4 (C = S). HRMS-TOF: *m/z* $[M + H]^+$ 679.0871 (calcd for $C_{26}H_{19}F_{12}N_4S_2$: 679.0854).

*1,1′-(1,3-phenylenebis(methylene))bis(3-(4-methoxyphenyl) thiourea) (**16**)*

White solid. 79%. Mp 169–170 °C. IR (UATR) cm^{-1}: 3256, 3219, 1542, 1511, 1299, 1221, 1032, 974, 830. ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 3.71 (s, 6H, 2 × OCH_3), 4.70 (d, $J = 6.0$ Hz, 4H, 2 × CH_2), 6.89 (d, $J = 8.8$ Hz, 4H, Ar*H*), 7.15–7.31 (m, 8H, Ar*H*), 7.93 (s, 2H, N*H*), 9.42 (s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 47.7 (2 × CH_2), 55.7 (2 × OCH_3), 114.5 (2 × C-3′, 2 × C-5′), 126.3 (C-4, C-6), 126.6 (C-2, 2 × C-2′, 2 × C-6′), 128.6 (C-5), 132.1 (2 × C-1′), 139.7 (C-1, C-3), 157.1 (2 × C-4′), 181.6 (C = S). HRMS-TOF: *m/z* $[M + H]^+$ 467.1564 (calcd for $C_{24}H_{27}N_4O_2S_2$: 467.1570).

*1,1′-(1,3-phenylenebis(methylene))bis(3-(4-bromophenyl) thiourea) (**17**)*

White solid. 80%. Mp 135–136 °C. IR (UATR) cm^{-1}: 3311, 3210, 1540, 1526, 1344, 1068, 825. ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.71 (s, 4H, CH_2), 7.17–7.33 (m, 4H, Ar*H*), 7.40 (d, $J = 8.8$ Hz, 4H, Ar*H*), 7.46 (d, $J = 8.8$ Hz, 4H, Ar*H*), 8.28 (s, 2H, N*H*), 9.70 (s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 47.2 (2 × CH_2), 116.2 (2 × C-4′), 125.3 (2 × C-2′, 2 × C-6′), 126.1 (C-4, C-6), 126.3 (C-2), 128.4 (C-5), 131.4 (2 × C-3′, 2 × C-5′), 138.7 (2 × C-1′), 139.0 (C-1, C-3), 180.9 (C = S). HRMS-TOF: *m/z* $[M + Na]^+$ 584.9371 (calcd for $C_{22}H_{20}Br_2N_4NaS_2$: 584.9388).

*1,1′-(1,3-phenylenebis(methylene))bis(3-(4-trifluoromethylphenyl)thiourea) (**18**)*

White solid. 73%. Mp 193–194 °C. IR (UATR) cm^{-1}: 3245, 1542, 1324, 1119, 1067, 840. ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.75 (d, $J = 4.8$ Hz, 4H, 2 × CH_2), 7.20–7.35 (m, 4H, Ar*H*), 7.61 (d, $J = 8.6$ Hz, 4H, Ar*H*), 7.70 (d, $J = 8.6$ Hz, 4H, Ar*H*), 8.47 (br.s, 2H, N*H*), 9.95 (br. s, 2H, N*H*). ^{13}C NMR (75 MHz, DMSO-d_6) δ 47.2 (2 × CH_2), 122.3 (2 × C-3′, 2 × C-5′), 123.7 (q, $^2J_{CF} = 32$ Hz, 2 × C-4′), 124.4 (q, $^1J_{CF} = 270$ Hz, CF_3), 125.7 (2 × C-2′, 2 × C-6′), 126.3 (C-4, C-6), 126.4 (C-2), 128.4 (C-5), 138.8 (C-1, C-3), 143.3 (2 × C-1′), 180.8 (C = S). HRMS-TOF: *m/z* $[M + H]^+$ 543.1086 (calcd for $C_{24}H_{21}F_6N_4S_2$: 543.1106).

Cytotoxic assay: cancer cell lines

Cells suspended in the corresponding culture medium were inoculated in 96-well microtiter plates (Corning Inc., NY, USA) at a density of 10,000–20,000 cells per well, and incubated for 24 h at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. An equal volume of additional medium containing either serial dilutions of the test compounds, positive control (etoposide and/or doxorubicin), or negative control [dimethyl sulfoxide (DMSO)] was added to the desired final concentrations, the microtiter plates were incubated for an additional 48 h. The number of surviving cells in each well was determined using MTT assay (Carmichael et al. 1987; Mosmann 1983) (for adherent cells: HuCCA-1, HepG2, and A549 cells) and XTT assay (Doyle and Griffiths 1997) (for suspended cells: MOLT-3 cells). The IC_{50} value is defined as the drug (or compound) concentration that inhibits cell growth by 50% (relative to negative control).

Antimalarial assay: radioisotope techniques

In vitro culture of *P. falciparum* (K1, multidrug resistant strain) was performed, according to (Trager and Jensen 1976), in RPMI 1640 medium containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 32 mM $NaHCO_3$ and 10% heat activated human serum with 3% erythrocytes, in humidified 37 °C incubator with 3% CO_2. The culture was passaged with fresh mixture of erythrocytes and medium every day to maintain cell growth. Antimalarial activity was determined by microculture radioisotope techniques as described by Desjardins et al. (1979) (Desjardins et al. 1979). Briefly, a mixture of 200 µL of 1.5% erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 µL of the medium containing a test sample dissolved in 1% DMSO (0.1% final concentration) for 24 h. Subsequently, 25 µL of $[^3H]$ hypoxanthine (Amersham, USA) in culture medium (0.5 µCi) was added to each well and the plates were incubated for an additional 24 h. Levels of incorporated radioactive labeled hypoxanthine, indicating parasite growth, were determined using the Top Count microplate scintillation counter (Packard, USA). The percentage of parasite growth was calculated using the signal count per minute of treated

CPMT and untreated conditions (CPMU) as shown by the following equation;

$$\%\,\text{Parasite growth} = \text{CPMT}/\text{CPMU} \times 100.$$

Cytotoxicity assay: normal cell line (Vero)

Cytotoxicity assay was performed using the green fluorescent protein (GFP) detection method (Hunt et al. 1999). African green monkey kidney (Vero, ATCC CCL-81) cell line was stably transfected with pEGFP-N1 plasmid (Clontech) for constructing GFP-expressing Vero cell. The cell line was propagated in a medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 0.8 mg/mL geneticin, and maintained at 37 °C in a humidified incubator with 5% CO_2. In brief, a 45 µL of cell suspension at 3.3×10^4 cells/mL was added to each well of 384 well plates containing 5 µL of tested compounds diluted in 0.5% DMSO. The plate was further incubated under the same condition for 4 days. Fluorescence signals were measured at excitation and emission wavelengths of 485 and 535 nm, respectively by using SpectraMax M5 microplate reader (Molecular Devices, USA). Fluorescence signal at day 4 was substrated with background fluorescence at day 0. IC_{50} values were determined from dose-response curves. Ellipticine and 0.5% DMSO were used as a positive and a negative control, respectively.

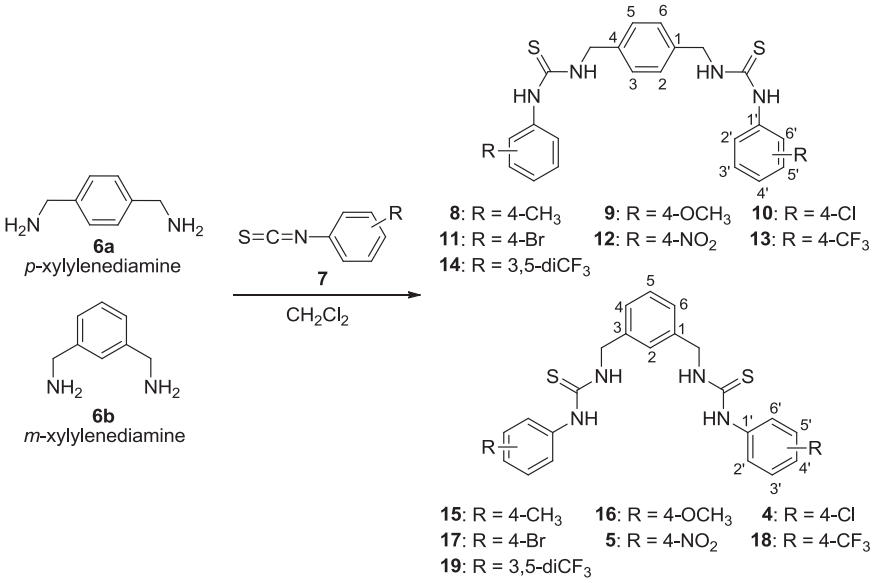
Antimicrobial assay: agar dilution

Agar dilution method was employed to investigate antimicrobial properties of the tested compounds as described previously (Prachayasittikul et al. 2011). Briefly, tested compounds dissolved in DMSO were mixed with Müller Hinton (MH) broth and added to MH agar for preparing their serial dilutions at the final concentrations of 0.5–256 µg/mL. The DMSO without the tested compounds was tested in parallel with the compounds. The MH broth was the negative control, whereas ampicillin and ciprofloxacin were used as reference drugs. Twenty-eight strains of microorganisms as shown below were cultured in MH broth at 37 °C for 24 h. The microbial cultures were individually adjusted the cell suspension to 0.5 McFarland standards (1.5×10^8 CFU/mL) using 0.9% normal saline solution. The inoculums of microorganisms were dropped onto the prepared plate using multipoint inoculator and incubated at 37 °C for 18–48 h. After the incubation, the lowest concentration of each tested compound which inhibited the visible growth of microorganisms was reported as MIC. Twenty-eight strains of tested microorganisms were sixteen Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Serratia marcescens* ATCC 8100, *Salmonella typhimurium* ATCC 13311, *Salmonella choleraesuis* ATCC 10708, *Shewanella putrefaciens* ATCC 8071, *Achromobacter xylosoxidans* ATCC 2706, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas stutzeri* ATCC 17587, *Shigella dysenteriae*, *Salmonella enteritidis, Morganella morganii*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Plesiomonas shigelloides*; ten Gram-positive-bacteria: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* ATCC 33186, *Micrococcus luteus* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes*, *Bacillus cereus*, and two diploid fungi (yeast): *Candida albicans* ATCC 90028, and *Saccharomyces cerevisiae* ATCC 2601.

Molecular docking

Molecular docking was performed to investigate possible binding modalities of the synthesized ligands toward their possible targets. Crystal structures of the target proteins were downloaded from RSCB protein data bank (http://www.rcsb.org/). The studied targets for anticancer and antimicrobial activities include α-tubulin and β-tubulin complex (PDB id 1SA0) from *Bos Taurus* in co-crystallized form with colchicines and DNA topoisomerase IV from *Staphylococcus aureus* in co-crystallized form with novobiocin (PDB id 4URN), respectively. The target protein structures were prepared by adding essential hydrogen atoms and repairing missing side chains using the WHAT IF web server version 10.1 (Vriend 1990). Non-polar hydrogen atoms were merged, Gasteiger atomic charges were assigned, and atom type of the protein structures were specified using AutoDock Tools version 1.5.6. (Morris et al. 2009; Sanner 1999). The investigated bis-thiourea ligands were constructed using Marvin Sketch version 6.1.4 (ChemAxon 2013) and were geometrically optimized by Gaussian 09 (Frisch et al. 2009) using Becke's three-parameter hybrid method with the Lee–Yang–Parr correlation functional (B3LYP) together with the 6-31 g(d) basis set. All ligands were prepared by merging non-polar hydrogen atoms, defining rotatable bonds and assigning partial atomic charge using AutoDock Tools version 1.5.6 (Morris et al. 2009; Sanner 1999). Grid boxes for tubulin (25 Å × 25 Å × 25 Å) and topoisomerase (25 Å × 25 Å × 25 Å) were generated to intend searching modality. Center of the receptor binding site on grid boxes were allocated using x, y and z coordinates of 118.36, 90.56, and 5.25 for tubulin, and −50.33, −8.80 and −29.79 for topoisomerase IV. AutoDock Vina, as a part of the PyRx 0.8 software (Dallakyan 2013), was used to perform the molecular docking simulation. The co-crystallized ligands were re-docked to their target proteins to validate docking protocol

Scheme 1 Synthesis of bis-thioureas (**4**, **5**, **8–19**) from xylylenediamines **6** and isothiocyanates **7**



(i.e., colchicine for α-tubulin and β-tubulin complexes and novobiocin for DNA topoisomerase IV). Re-docked results were evaluated by calculation of root mean standard deviation (RMSD) using Chimera software (Pettersen et al. 2004). Docking poses of the investigated compounds were visualized using PyMOL (Delano 2002) and two-dimensional ligand-protein interaction diagrams were generated using Discovery Studio Visualization version 16.1.0.15350 (BioVia 2017).

Results and discussion

Chemistry

Two sets of bis-thiourea derivatives constituting various substitutuents ($R = CH_3$, OCH_3, Cl, Br, NO_2, CF_3) at position-4 and position-3,5 ($R = diCF_3$) on phenyl ring, a *para* series (**8–14**) and a *meta* series (**4**, **5** and **15–19**), were readily synthesized by treatment of (*p*- or *m*-) xylylenediamines **6** with the corresponding isothiocyanates **7** in dichloromethane (Scheme 1). Structures of all obtained bis-thioureas (**4**, **5** and **8–19**) were characterized by ^{1}H NMR, ^{13}C NMR, IR, and HRMS. Typically, infrared spectra of the synthesized compounds had strong N–H absorptions (3202–3375 cm^{-1}), and thiocarbonyl groups (C = S) showed stretching absorption band values in the range of 1050–1250 cm^{-1}. ^{1}H NMR spectra exhibited two singlets at δ 7–11 ppm, which were assigned to the N–H protons. ^{13}C NMR spectral data analysis of thioureas showed a typical signal for thiocarbonyl carbon in the range of δ 180–182 ppm. In^{13}C NMR, compounds **13**, **14**, **18** and **19** revealed the distinctive peaks as two quartets with coupling constant of 269–272 Hz ($^1J_{CF}$) and 32 Hz ($^2J_{CF}$), which proved the presence of CF_3 moiety and the carbon adjacent to CF_3 group, respectively.

Biological activities

Cytotoxic activity

Cytotoxicity of the synthesized bis-thiourea derivatives was examined against human cancer cell lines; HuCCA-1 (cholangiocarcinoma), HepG2 (hepatocellular carcinoma), A549 (lung carcinoma) and MOLT-3 (lymphoblastic leukemia), as summarized in Table 1. These compounds were also evaluated toward non-cancerous, Vero cell line derived from African green monkey kidney (Table 1), and a selective index was calculated as provided in Table S1.

In the *para* derivatives (**8–14**), compound **8** with methyl substituent ($R = CH_3$) displayed cytotoxic activity against HuCCA-1 and HepG2 cells with IC_{50} values of 82.83 and 21.67 μM, respectively. Replacement of the methyl group with methoxy, chloro and bromo ($R = OCH_3$, Cl, Br) substituents led to compounds (**9–11**) with a total loss of cytotoxicity. However, compounds **12** and **13** obtained by the replacement of methyl group of compound **8** with nitro ($R = NO_2$) and trifluoromethyl ($R = CF_3$) moieties showed inhibitory potencies against HepG2 and MOLT-3 cell lines. Significantly, an introduction of two trifluoromethyl groups ($R = 3,5\text{-}diCF_3$) on the phenyl ring provided the analog **14** which displayed a broad spectrum of anticancer activity toward all of the tested cells with IC_{50} values in the range of 2.49–30.95 μM, and had a selective index in the range of

Table 1 Cytotoxic and antimalarial activities (IC_{50}, μM) of bis-thiourea derivatives (**4**, **5**, **8–19**)

Compound	Cancer cell lines[a]				Antimalarial[b]	Vero cell line[c]
	HuCCA-1	HepG2	A549	MOLT-3		
8	82.83 ± 8.48	21.67 ± 2.24	Non-cytotoxic	Non-cytotoxic	Inactive	Non-cytotoxic
12	Non-cytotoxic	61.02 ± 2.56	Non-cytotoxic	11.40 ± 0.45	Inactive	Non-cytotoxic
13	Non-cytotoxic	15.52 ± 1.12	Non-cytotoxic	6.06 ± 0.79	17.95	Non-cytotoxic
14	30.95 ± 4.58	8.81 ± 0.71	26.16 ± 0.35	2.49 ± 0.19	Inactive	70.81
15	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	13.90 ± 1.09	Inactive	33.50
16	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	32.32 ± 6.92	Inactive	Non-cytotoxic
4	30.22 ± 1.25	13.29 ± 1.05	Non-cytotoxic	2.23 ± 0.26	5.09	2.84
17	non-cytotoxic	41.30 ± 0.03	Non-cytotoxic	2.62 ± 0.28	2.80	3.28
5	14.84 ± 0.50	10.53 ± 0.58	44.71 ± 3.49	3.40 ± 0.44	4.29	11.24
18	14.47 ± 1.38	8.40 ± 0.43	17.97 ± 2.96	1.55 ± 0.17	1.92	13.18
19	18.82 ± 2.34	1.50 ± 0.23	16.67 ± 1.11	3.63 ± 0.46	3.33	30.90
Etoposide[d]	ND	26.05 ± 0.50	ND	0.041 ± 0.003	ND	ND
Doxorubicin[d]	0.42 ± 0.02	0.57 ± 0.05	0.37 ± 0.02	ND	ND	ND
Mefloquine[d]	ND	ND	ND	ND	0.0247	ND
Dihydroartemisinine[d]	ND	ND	ND	ND	0.0019	ND
Ellipticine[d]	ND	ND	ND	ND	ND	2.16

$IC_{50} > 50$ μg/mL denoted as non-cytotoxic. $IC_{50} > 10$ μg/mL denoted as inactive. ND = not determined

[a] Cancer cell lines comprise the following: HuCCA-1 human cholangiocarcinoma cancer cell line, HepG2 human hepatocellular carcinoma cell line, A549 human lung carcinoma cell line, MOLT-3 human lymphoblastic leukemia cell line

[b] Antimalarial against *Plasmodium falciparum*

[c] Vero cell line was African green monkey kidney cell line

[d] Etoposide, doxorubicin, mefloquine, dihydroartemisinine and ellipticine were used as reference drugs

2.29–28.44. Notably, the derivatives **8**, **13** and **14** were shown to be more potent anticancer agents against HepG2 cell compared with etoposide, the reference drug.

In the *meta* derivatives (**4**, **5**, **15**-**19**), results showed that all of the tested molecules exerted significant cytotoxicity, especially against MOLT-3 cancer cell line (IC_{50} = 1.55–32.22 μM). Methyl compound **15** and methoxy analog **16** selectively inhibited MOLT-3 cell line with IC_{50} values of 13.90 and 32.32 μM, respectively. Improvement of cytotoxic activity against HepG2 and MOLT-3 cells was observed when the molecules contained halogen substituents (R = Cl and Br) as seen in compounds **4** and **17**. Furthermore, the chloro analog **4** also inhibited HuCCA-1 cell line with IC_{50} of 30.22 μM. It should be noted that the substitution with strong electron withdrawing groups (R = NO_2 and CF_3) led to the derivatives (**5**, **18** and **19**) which enhanced inhibitory potency with a wide range of anticancer activity against all tested cells. Apparently, trifluoromethyl analog **18** was shown to be the most potent cytotoxic compound against HuCCA-1 and MOLT-3 cells with IC_{50} of 14.47 and 1.55 μM, respectively. Moreover, bis-trifluoromethyl compound **19** displayed the most potent cytotoxic activity against HepG2 and A549 cells with IC_{50} values of 1.50 and 16.67 μM, respectively. Derivative **19** was the promising compound showing the best safety profile with selective index (SI) values of 1.64–20.60. Interestingly, derivatives **4**, **5**, **18** and **19** exhibited higher anticancer activity against HepG2 cell than that of the etoposide. Among these, compound **19** (SI = 20.60) was the most potent cytotoxic agent (HepG2) with 17.4-fold stronger potency than the etoposide, but with 2.6-fold weaker potency than the doxorubicin. Clearly, most of the meta-analogs with $R = NO_2$, CF_3 and diCF_3 were more active than the corresponding *para*-counterparts as seen in compounds **5** > **12**, **18** > **13** and **19** > **14**. Considering isomeric and substituent effects, it could be presumed that an orientation of bis-thiourea groups in *meta*-position on phenyl ring A afforded the compounds that better occupy at the target sites of action compared with *para*-isomers. Along this line, electron withdrawing groups can create ionic charge on N-atom of the thiourea moiety that may be required for interacting with the sites of action.

Antimalarial activity

Antimalarial activity of the bis-thioureas (**4, 5, 8**-**19**) was assayed against *Plasmodium falciparum* (K1, multidrug resistant strain) as shown in Table 1.

Trifluoromethyl derivative (**13**) was the only compound in the *para* series which exhibited antimalarial activity with an IC_{50} of 17.95 µM. It was found that *meta*-thioureas (**15** and **16**)-bearing electron donating groups ($R = CH_3$ and OCH_3) were shown to be inactive compounds, while the *meta*-thioureas (**4**, **5**, **17–19**) containing electron withdrawing groups ($R =$ Cl, Br, NO_2, CF_3, diCF_3) displayed significant antimalarial activity with IC_{50} values in the range of 1.92–5.09 µM. The order of decreasing inhibitory potency of these thioureas based on the substituent effects (*R*) as shown by the following trend: $CF_3 > Br > 2 \times CF_3 > NO_2 >$ Cl. Notably, compounds **18** and **19** were found to be the most promising compounds that exerted antimalarial activity with the best safety index (SI = 6.86 and 9.28, respectively). The *meta*-compound **18** with $R = CF_3$ showed 9-fold more active than the corresponding *para*-compound **13** ($R = CF_3$). At this point, it is reasonable to conclude that the presence of lipophilic trifluoromethyl moiety at the 4-position on the phenyl rings B of thiourea groups plays an important role in the inhibition of *P. falciparum* as indicated by the most potent compounds **13** and **18** in the *para*-series and *meta*-series, respectively.

Antimicrobial activities

All bis-thiourea derivatives (**4, 5, 8-19**) were tested for antimicrobial activity, using the agar dilution method, against 28 strains of microorganisms including 16 Gram-negative bacterial, 10 Gram-positive bacterial and 2 fungal strains. Results (Table 2) showed that most of active antimicrobial compounds were Cl, NO_2, CF_3 and diCF_3 derivatives (**4**, **5**, **18**, **19**) in the *meta*-series, except for compounds **10** and **12** (*para*-series).

The chloro derivatives in both *meta*-series and *para*-series (**4** and **10**) showed selective complete growth inhibition against Gram-positive bacteria, *M. luteus* ATCC 10240 with a MIC of 538.43 µM. The nitro analog **12** displayed antigrowth activity against Gram-negative bacteria, *P. shigelloides* and *A. xylosoxidans* ATCC 2706 with MIC values of 257.77 and 515.55 µM, respectively. However, *A. xylosoxidans* ATCC 2706 was partially inhibited (25%) by the nitro compound **5** (*meta*-series) at 515.55 µM. Mostly, the *para*-analog with NO_2 substituent (**12**) exerted complete inhibition against Gram-positive bacteria such as *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *M. luteus* ATCC 10240, *B. subtilis* ATCC 6633, *L. monocytogenes* and *B. cereus* with the MIC value of 257.77 µM. However, *S. epidermidis* ATCC 12228 and fungus, *S. cerevisiae* ATCC 2601 were inhibited by the analog **12** with the MIC of 515.55 µM. Trifluoromethyl (**18**) and bis-trifluoromethyl (**19**) derivatives displayed a broad spectrum of antimicrobial activity against the same strains of Gram-positive bacteria and fungi. It was observed that compound **19** showed more potent antimicrobial activity than that of compound **18**. Similarly, compound **19** displayed higher activity against Gram-negative bacteria including *S. dysenteriae* and *P. shigelloides*. However, compound **19** did not show growth inhibition against *A. xylosoxidans* ATCC 2706 while compound **18** exerted the activity with MIC value of 471.84 µM.

Promisingly, bis-trifluoromethyl derivative **19** was the most potent compound in the *meta*-series. Compound **19** had 1.8 and 3.7-fold higher potency against Gram-negative bacteria (*S. dysenteriae* and *P. shigelloides*) than that of the reference drug, ampicillin. Mostly, compound **19** inhibited the growth of Gram-positive bacteria and fungi better than the ampicillin. Moreover, compound **19** possessed the lowest MIC value of 1.47 µM against *S. epidermidis* ATCC 2228 and *M. luteus* ATCC 10240, which were 1.8 and 7.3 fold more potent than the ampicillin, respectively. Interestingly, compound **19** showed comparable activity to ciprofloxacin against *S. dysenteriae*, *P. shigelloides*, *M. luteus* ATCC 10240 and *L. monocytogenes*.

The antimicrobial activity revealed that bis-thioureas **12**, **18** and **19** exhibited selective activity against Gram-positive bacteria and fungi. Results are in accordance with the previous report of thiourea derivatives (Cunha et al. 2007). It should be noted that the position of substituents (*R*) and electronegativity of fluorine atom play an important role in the observed antimicrobial activity. There are many CF_3-containing drugs have been approved by the food and drug administration (Zhu et al. 2014; Wang et al. 2014). The role of trifluoromethyl function is recognized in medicinal chemistry as a substituent of distinctive properties such as enhancing efficacy by promoting electrostatic interactions with targets, improving cellular membrane permeability due to its lipophilicity and increasing strength toward oxidative metabolism of the drug (Müller et al. 2007; Purser et al. 2008; Böhm et al. 2004; Smart 2001).

Molecular docking

As previously mentioned, the anticancer and antimicrobial activities of the lead thioureas **2** (Shing et al. 2014) and **1** (Bielenica et al. 2015) are associated with their abilities to inhibit tubulin and topoisomerase IV, respectively. Furthermore, many (thio)urea derivatives are known inhibitors of the two putative targets, which have been supported by the literatures (Li et al. 2009; Shing et al. 2014; Bielenica et al. 2015; Azam et al. 2015; Ehmann and Lahiti 2014; Grillot et al. 2014; Basarab et al. 2013; Charifson et al. 2008). Molecular docking was performed to investigate binding modalities of the synthesized bis-thiourea derivatives against target proteins, i.e., tubulin (anticancer activity) and topoisomerase IV (antimicrobial activity). Due to the lack of literature specifying the target proteins for antimalarial activity of the compounds in this class, thus,

Table 2 Antimicrobial activity (MIC) of the bis-thiourea derivatives (**4**, **5**, **8–19**)

Microorganisms	MIC (μM)							
	10	**12**	**4**	**5**	**18**	**19**	Ampicillin[a]	Ciprofloxacin[a]
Gram-negative bacteria								
E. coli ATCC 25922	I	I	I	I	I	I	21.54	<0.32
K. pneumoniae ATCC 700603	I	I	I	I	I	I	I	0.32
S. marcescens ATCC 8100	I	I	I	I	I	I	I	<0.32
S. typhimurium ATCC 13311	I	I	I	I	I	I	1.35	<0.32
S. choleraesuis ATCC 10708	I	I	I	I	I	I	I	0.32
S. putrefaciens ATCC 8071	I	I	I	I	I	I	1.35	<0.32
A. xylosoxidans ATCC 2706	I	515.55	I	515.55[b]	471.84	I	I	10.37
P. aeruginosa ATCC 15442	I	I	I	I	I	I	I	1.30
P. aeruginosa ATCC 27853	I	I	I	I	I	I	I	2.59
P. stutzeri ATCC 17587	I	I	I	I	I	I	0.67	<0.32
S. dysenteriae	I	I	I	I	14.74	2.95	5.39	2.59
S. enteritidis	I	I	I	I	I	I	2.69	<0.32
M. morganii	I	I	I	I	I	I	I	<0.32
A. hydrophila	I	I	I	I	I	I	10.77	<0.32
C. freundii	I	I	I	I	I	I	21.54	<0.32
P. shigelloides	I	257.77	I	I	3.69	2.95	10.77	2.59
Gram-positive bacteria								
S. aureus ATCC 29213	I	257.77	I	I	3.69	2.95	0.34	1.30
S. aureus ATCC 25923	I	257.77	I	I	3.69	2.95	0.34	1.30
S. epidermidis ATCC 12228	I	515.55 257.77[c]	I	I	3.69	1.47	2.69	<0.32
E. faecalis ATCC 29212	I	515.55[c]	I	I	29.49	5.89	21.54	1.30
E. faecalis ATCC 33186	I	515.55[c]	I	I	29.49	5.89	21.54	2.59
M. luteus ATCC 10240	538.43	257.77	538.43	I	3.69	1.47	10.77	1.30
C. diphtheriae NCTC 10356	I	I	I	I	14.74	5.89	10.77	2.59
B. subtilis ATCC 6633	I	257.77	I	I	3.69	2.95	1.35	<0.32
L. monocytogenes	I	257.77	I	I	3.69	2.95 1.47[d]	<0.34	2.59
B. cereus	I	257.77	I	I	3.69	2.95	43.08	0.32
Fungi								
C. albicans ATCC 90028	I	I	I	I	7.37	2.95	5.39	1.30
S. cerevisiae ATCC 2601	I	515.55	I	I	14.74	2.95	5.39	1.30

Minimum inhibitory concentration (MIC) was the lowest concentration that inhibited the growth of microorganisms

MIC > 256 μg/mL was indicated as an inactive (I)

[a] Ampicillin and ciprofloxacin were used as reference drugs

[b] At 515.55 μM, compound **5** showed 25% inhibition against *A. xylosoxidans* ATCC 2706

[c] At 515.55 μM and 257.77 μM, compound **12** showed 25% inhibition against *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 33186 and 75 % inhibition against *S. epidermidis* ATCC 12228, respectively

[d] At 1.47 μM, compound **19** showed 75% inhibition against *L. monocytogenes*

molecular docking against antimalarial target was excluded from this study. Co-crystallized ligands of tubulin (i.e., colchicine) and topoisomerase IV (i.e., novobiocin) were re-docked to their target protein structures to validate the docking protocols. Re-docking poses of colchicine to tubulin (RMSD = 0.614 A°), and novobiocin to topoisomerase IV (RMSD = 0.388 A°) are shown in Fig. S1A, B, respectively. The calculated RMSD values indicated that the docking protocols are acceptable for the analysis of possible binding modes of the investigated compounds. All bis-thiourea derivatives (**4**, **5**, **8–19**) were docked to tubulin and topoisomerase IV structures (Fig. S2A, S2B, respectively). Results suggested that all bis-thiourea analogs possibly bind to the target proteins via the same binding

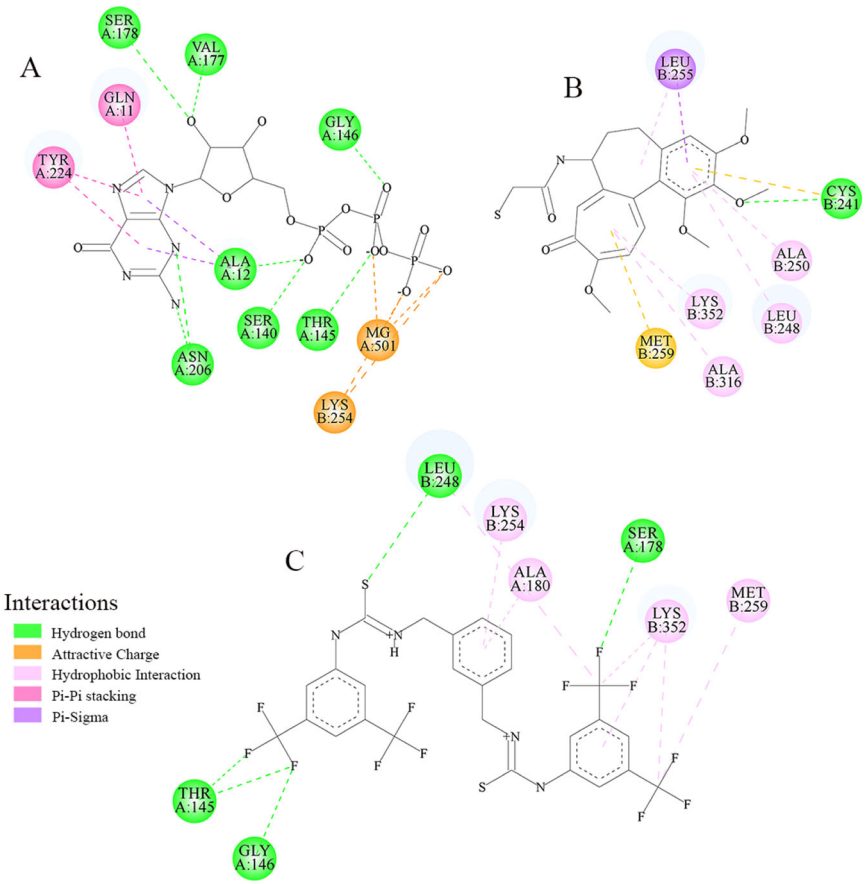


Fig. 3 Two-dimensional ligand–protein interaction diagrams of GTP (**a**) colchicine (**b**) and compound **19** (**c**) with tubulin

sites of the co-crystallized ligands i.e., colchicine binding site on the β-tubulin (Fig. S2A) and novobiocin binding site on the topoisomerase IV (Fig. S2B). Binding energy of the investigated compounds against the target proteins, tubulin and topoisomerase IV, are in the range of −7.7 to −10.5 and −6.6 to −7.7 kcal/mol, respectively (Table S2). Docking results of the topoisomerase IV demonstrated that all of the investigated bis-thioureas exhibited weaker binding affinities than the co-crystallized ligand, novobicin (−9.8 kcal/mol, Table S2). In contrast, a promising potential of the compounds in this class was highlighted against tubulin target as the stronger binding affinities observed for three bis-thioureas **14** (−10.5 kcal/mol), **18** (−9.8 kcal/mol) and **19** (−10.3 kcal/mol), compared with the co-crystallized ligand, colchicine (−9.5 kcal/mol, Table S2). Furthermore, the promising compound **19** with highly potent cytotoxic activity (Table 1) and selectivity index (Table S1) was selected as a model for elucidating the ligand-protein binding interactions. Two-dimensional binding interaction diagrams of tubulin to co-crystalize ligands, i.e., guanosine-5′-triphosphate (GTP, Fig. 3a) and colchicine (Fig. 3b) as well as compound **19** (Fig. 3c) are shown in Fig. 3. Considering the tubulin binding, fluorine (F) and sulfur (S) atoms play role in the formation of hydrogen bonds, using F atom of CF_3 group with THR145, GLY146 and SER178 residues on the α-chain as well as using S atom of thiourea moiety with LEU248 residue on the β-chain of tubulin complex. Hydrophobic interactions (Fig. 3c) also take part in the binding between phenyl rings and CF_3 moieties of the compound **19** via several residues of both α-chain and β-chain (i.e., ALA 180 of α-chain as well as LYS254, MET259 and LYS352 of β-chain). Moreover, the docking post of compound **19** suggested that this compound has several common binding sites with co-crystallized ligands found in the tubulin. GTP found in the binding pocket of α-chain tubulin also formed hydrogen bondings with THR145, GLY146 and SER178 (Fig. 3a) as observed in the docking pose of compound **19**. Furthermore, colchicine that occupied the binding site of β-chain also shared the common binding residues with compound **19**. Colchicine interacted with β-chain tubulin via attractive charge and hydrophobic interaction with MET259 and LYS352, respectively (Fig. 3b) whereas compound **19** formed hydrophobic interaction with these mentioned residues. This circumstance suggested that compound **19** might prevent multimerization of tubulin by acting as the dual site blocker to the binding sites of GTP and colchicine. Additionally, two-dimensional binding interaction diagrams of

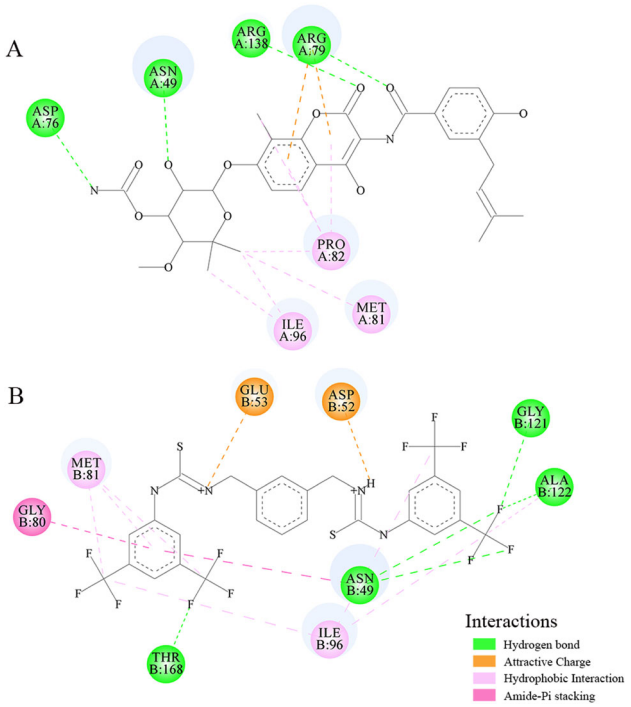


Fig. 4 Two-dimensional ligand–protein interaction diagrams of novobiocin (**a**) and compound **19** (**b**) with topoisomerase IV

topoisomerase IV to novobiocin and compound **19** are shown in Fig. 4. Likewise, the compound **19** binds to topoisomerase IV (Fig. 4b) via the formation of hydrogen bonds (ASN49, GLY121, ALA122 and THR168 residues) and hydrophobic interactions (MET81 and ILE96 residues). In addition, attractive charge interaction and amide-pi stacking may be essential for the binding of compound **19** with topoisomerase IV as noted for the charge interaction of positively charged nitrogen (N^+) atoms with ASP52 and GLU53 residues including the amide-pi stacking with GLY80 residue (Fig. 4b). Interestingly, the common characteristics of compound **19** and novobiocin bindings to topoisomerase IV were observed. Both compounds can form hydrogen bond with ASN49 as well as hydrophobic interactions with the pockets of ILE96 and MET81. This suggested that the compound **19** could possibly inhibit topoisomerase IV by the similar mechanism of novobiocin.

Conclusions

Fourteen bis-thiourea derivatives (**4, 5, 8–19**) have been successfully synthesized by a simple and high yield reaction as well as an inexpensive manner derived from xylylenediamines and phenylisothiocyanates. Cytotoxic activity evaluation exposed that most of the thioureas displayed cytotoxicity against MOLT-3 cell line. Compounds (**4**, **5**, **8**, **13**, **14**, **18** and **19**) displayed higher cytotoxic potency against HepG2 cells than the control drug, etoposide. In particular, the *meta*-thiourea containing bis-trifluoromethyl moiety (**19**) was the promising potent cytotoxic agent (IC_{50} = 1.50–18.82 μM) with the best safety index (1.64–20.60). Antimalarial activity results fulfilled that the trifluoromethyl derivative **18** was shown to be the most potent compound (IC_{50} = 1.92 μM, SI = 6.86). Significantly, the bis-trifluoromethyl derivative **19** was also shown to be the most potent antimicrobial agent. The compound **19** displayed higher potency than that of the ampicillin against Gram-negative bacteria: *S. dysenteriae* and *P. shigelloides*; Gram-positive bacteria: *S. epidermidis* ATCC 12228, *E. faecalis* ATCC 29212, *E. faecalis* ATCC 33186, *M. luteus* ATCC 10240, *C. diphtheriae* NCTC 10356, *B. cereus* and fungi: *C. albicans* ATCC 90028 and *S. cerevisiae* ATCC 2601. Promisingly, the potency of compound **19** against *S. dysenteriae*, *P. shigelloides*, *M. luteus* ATCC 10240 and *L. monocytogenes* was comparable to that of ciprofloxacin. The molecular docking revealed that trifluoromethyl moiety and thiourea group of the investigated compounds play roles in binding interaction with the target proteins. It should be noted that the high electronegativity atoms, a relatively small size and tightly bound of trifluoromethyl moiety are essential features for anticancer, antimalarial and antimicrobial activities of the compounds. In conclusion, the findings suggested that the bis-thioureas with trifluoromethyl group(s) (**18** and **19**) are promising compounds to be further developed as novel anticancer, antimalarial and antimicrobial agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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