Synthesis, cytotoxic, proapoptotic evaluation, and molecular docking study of some new *N*-substituted sulfonyl-3-indolyl heterocycles

Eslam R. El-Sawy[a], Heba M. Abo-Salem[a], Shaymaa M. Yahya[b], Manal S. Ebaid[a], Adel H. Mandour[a]

[a]Department of Natural Compounds Chemistry, [b]Department of Hormones, National Research Centre, Dokki, Giza, Egypt

Correspondence to Eslam Reda El-Sawy, Chemistry Department of Natural Compounds, National Research Centre, Dokki 12311, Giza, Egypt
Tel: +20 122 551 1372; fax: +20 333 70931; e-mail: eslamelsawy@gmail.com

Received 06 August 2014
Accepted 09 September 2014

Egyptian Pharmaceutical Journal
2015, 14:15–29

Background and objectives
Apoptosis, also called programmed cell death, is a fundamental biological phenomenon that plays a crucial role in processes such as immune regulation, embryogenesis, and general tissue homeostasis. B-cell lymphocyte/leukemia-2 (*BCL*-2) family members are key regulators of apoptosis. The ability of the indole derivative to disrupt microtubule assembly and induce G2/M arrest, polyploidy, and apoptosis through mitochondrial pathways in COLO 205 cell has been reported; in addition, it reduced the levels of procaspase-3, procaspase-9, *BCL*-xL, and *BCL*-2 gene. The aim of this study is to describe the synthesis of some new *N*-substituted sulfonyl-3-indolyl heterocycles and to study their cytotoxic and proapoptotic effects. In addition, a molecular docking study of the most biologically active compounds against the *BCL*-2 protein is discussed.

Materials and methods
A new series of triazolopyridines **3a-c**, diaminopyridines **4a–c**, acetamides **5a–c**, triazolo[1,5-*a*] pyridines **6a–c–8a–c**, pyrido[1,2-*b*][1,2,4]triazines **9a–c**, **10a–c**, pyrazoles **11a–c, 12a–c**, and pyrimidine derivatives **13a–c–15a–c** were prepared by an initial reaction of 2-((*N*-substituted sulfonyl-1*H*-indol-3-yl)methylene) malononitriles **2a–c** with different reagents. The newly synthesized compounds were tested for their cytotoxic activity against the HepG2 cell line. The compounds that showed promising IC_{50} values were chosen for the study of their proapoptotic effect on the *BCL*-2 gene, which is an antiapoptotic factor, and they significantly inhibited the expression levels of the *BCL*-2 gene. The binding mode of the most promising proapoptotic compounds was assessed by docking studies with the CHIMAERIC *BCL*2-XL protein (PDB ID: 2W3L).

Results and conclusion
From the data obtained, the most active compounds against the HepG2 cancer cell line were in the descending order of **10b>4c>10a>10c**, whereas compounds **3c**, **6c**, **9c**, **4b**, and 5c showed moderate to slight growth inhibition. Compounds **4c**, **5c**, **9c**, **10a**, **10b**, and **10c** significantly inhibited the expression levels of the *BCL*-2 gene. Docking results showed that compounds **5c** and **9c** showed good fitting within the pocket site of the protein molecular surface and had a minimum binding energy of –20.29 and –18.98 kJ/mol, respectively, in comparison with the co-crystallized ligand, which is in agreement with the experimental result of a proapoptotic effect.

Keywords:
cytotoxic and proapoptotic activities, indole-3-carboxaldehyde, molecular docking, pyrazole, pyridine, pyrimidine

Egypt Pharm J 14:15–29

1687-4315

Introduction

Apoptosis, also called programmed cell death, is a fundamental biological phenomenon that plays a crucial role in processes such as immune regulation, embryogenesis, and general tissue homeostasis. B-cell lymphocyte/leukemia-2 (*BCL*-2) family members are key regulators of apoptosis. The first group of genes in this family, which contains the *BCL*-2 gene and the *BCL*-2-like 1 isoform gene (*BCL*-xL), possesses antiapoptotic activity. The second group, which is comprised of proteins such as the *BCL*-2-associated X protein gene (Bax) and the *BCL*-2-antagonist killer gene (Bak), promotes cell death, thus carrying proapoptotic activity. The *BCL*-2 gene was discovered by virtue of its translocation into the immunoglobulin heavy-chain locus in follicular B-cell lymphoma [1]. *BCL*-2 gene, which is expressed in certain tumor cells, was identified as an important factor in regulating apoptosis [2]. Overexpression of the *BCL*-2 gene has been shown to promote cell survival [3]. However, functionalized nitrogen heterocycles play a predominant role in medicinal chemistry and they have been used intensively as scaffolds for drug development. The pyridine, pyrimidine, and pyrazole nucleus are known for their pronounced pharmaceutical activities namely antitumor, anti-inflammatory, and antimicrobial

activities [4–12]. In addition, indole which is the potent basic pharmacodynamic nucleus, has been reported to possess a wide variety of biological properties namely, antimicrobial, anti-inflammatory, anticancer, and antioxidant [13–17]. In the present work and in continuation of our search [18–21] for the preparation of new polyheterocycles with pharmaceutical value, here, we report the synthesis of some new *N*-substituted sulfonyl-3-indolyl heterocycles and study their cytotoxic and proapoptotic effects. In addition, molecular docking studies of the most biologically active compounds against *BCL-2* protein have been carried out.

Experimental

Chemistry

Melting points were determined in open capillary tubes on an Electrothermal 9100 digital melting point apparatus (serial No. 8694; Electrothermal Engineering Ltd, Rochford, UK) and were uncorrected. Elemental analyses were carried out on a Perkin-Elmer 2400 analyzer (Perkin-Elmer, Waltham, Massachusetts, USA) and were found to be within ±0.4% of the theoretical values. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer in KBr discs. The ^{1}H NMR spectra were measured using a Bruker Avance digital spectrometer 500 MHz (BRUKER BioSpin GMBH, Rheinstetten, Germany) in dimethyl sulfoxide (DMSO)-d_6, and chemical shifts were recorded in δ ppm relative to trimethylsilane as an internal standard. Mass spectra (EI) were run at 70 eV using a JEOL-JMS-AX500 mass spectrometer (Jeol Ltd, Tokyo, Japan). *N*-methylsulfonyl (**1a**), *N*-benzenesulfonyl (**1b**) *N*-(4-chlorobenzenesulfonyl) indole-3-carboxaldehydes (**1c**), and 2-(*N*-benzenesulfonyl-1*H*-indol-3-ylmethylene) malononitrile (**2b**) were prepared as reported [22–25].

Synthesis of 2a and 2c

To a solution of **1a** or **1c** (0.01 mol) in absolute ethanol (10 ml) containing piperidine (0.2 ml), malononitrile (0.66 g, 0.01 mol) was added. The reaction mixture was stirred for 30 min at room temperature and the precipitate formed was filtered off, washed with absolute ethanol, air dried, and crystallized from absolute ethanol to yield **2a** or **2c**, respectively.

2-((*N*-(methansulfonyl)-1*H*-indol-3-yl)methylene) malononitrile **2a**; melting point 189–191°C; yield 96%. IR (KBr): υ = 2207 (CN), 1625 (C=C), 1386 and 1136 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 3.12 (s, 3H, SO_2CH_3), 7.13–7.40 (m, 4H, Ar-H), 7.63 (1*H*, s, CH=C), 8.08 (s, 1*H*, indolyl 2-H). $C_{13}H_9N_3O_2S$ (271.29): calcd. C 57.55; H 3.34; N 15.49; found C 57.42; H 3.25; N 15.38.

2-((*N*-(4-chlorophenylsulfonyl)-1*H*-indol-3-yl) methylene) malononitrile **2c**; melting point 208–110°C; yield 96%. IR (KBr): υ = 2195 (CN), 1575 (C=C), 1378 and 1154 (SO_2-N), 745 cm^{-1} (C-Cl). ^{1}H NMR (DMSO-*d6*) δ: 7.11–7.53 (m, 9H, Ar-H), 7.84 (1*H*, s, CH=C), 8.34 (s, 1*H*, indolyl 2-H). $C_{18}H_{10}ClN_3O_2S$ (367.81): calcd. C 58.78; H 2.74; N 11.42; found C 58.62; H 2.81; N 11.32.

Synthesis of 3a, 3b, and 3c

To a solution of compound **2a**, **2b**, or **2c** (0.02 mol) in absolute ethanol (50 ml) containing triethylamine (0.5 ml), 2'-acetyl-2-cyanoacetohydrazide (0.28 g, 0.02 mol) was added. The reaction mixture was heated under reflux for 20–24 h. The solvent was evaporated under vacuum to about half the bulk of its volume and set aside in a refrigerator overnight. The precipitate formed was filtered off, washed with water, air dried, and crystallized from acetonitrile.

7-(*N*-methanesulfonyl-1*H*-indol-3-yl)-2-methyl-5-oxo-3,5-dihydro[1,2,4] triazolo[1,5-a]pyridine-6,8-dicarbonitrile **3a**; melting point 93–95°C; yield 30%. IR (KBr): υ = 3326 (NH), 2210 (CN), 1730 (C=O), 1604 (C=N), 1566 (C=C), 1377 and 1158 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 2.17 (s, 3H, CH_3), 3.82 (s, 3H, SO_2CH_3), 7.12–7.48 (m, 4H, Ar-H), 8.12 (s, 1*H*, NH), 8.27 (s, 1*H*, indolyl 2-H). EI-MS: *m/z* (%) = 392 (M^+, 0.01). $C_{18}H_{12}N_6O_3S$ (392.39): calcd. C 55.10; H 3.08; N 21.42; found C 55.24; H 2.92; N 21.26.

7-(*N*-benzenesulfonyl-1*H*-indol-3-yl)-2-methyl-5-oxo-3,5-dihydro[1,2,4] triazolo[1,5-a]pyridine-6,8-dicarbonitrile **3b**; melting point 173°C; yield 25%. IR (KBr): υ = 3324 (NH), 2212 (CN), 1704 (C = O), 1620 (C=N), 1522 (C = C), 1368 and 1175 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 454 (M^+, 0.01). $C_{23}H_{14}N_6O_3S$ (454.46): calcd. C 60.79; H 3.11; N 18.49; found C 60.64; H 3.24; N 18.35.

7-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl)-2-methyl-5-oxo-3,5-dihydro [1,2,4]triazolo[1,5-a] pyridine-6,8-dicarbonitrile **3c**; melting point 141–143°C; yield 37%. IR (KBr): υ = 3325 (NH), 2212 (CN), 1703 (C=O), 1618 (C=N), 1522 (C=C), 1376 and 1176 (SO_2-N), 748 cm^{-1} (C-Cl). ^{1}H NMR (DMSO-*d6*) δ: 1.19 (s, 3H, CH_3), 7.16–8.13 (m, 8H, Ar-H), 8.26 (s, 1*H*, indolyl 2-H), 8.78 (s, 1*H*, NH). $C_{23}H_{13}ClN_6O_3S$ (488.91): calcd. C 56.50; H 2.68; N 17.19; found C 56.37; H 2.51; N 17.05.

Synthesis of compounds 4a, 4b, and 4c

A mixture of compound **2a**, **2b**, or **2c** (5.0 mmol) and 2-cyanoacetic acid hydrazide (0.49 g, 5.0 mmol) in dry ethanol (50 ml) containing piperidine (0.1 ml) was heated under reflux for 6–8 h. After cooling, the solid formed was filtered off, washed with water, air dried, and crystallized from absolute ethanol.

1,6-diamino-4-(*N*-methanesulfonyl-1*H*-indol-3-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile **4a**; melting point 126–128°C; yield 89%. IR (KBr): υ = 3429 and 3317 (NH_2), 2206 (CN), 1715 (C=O), 1652 (C=C), 1338 and 1174 cm^{-1} (SO_2-N). ^1H NMR (DMSO-*d6*) δ: 1.14 (s, 2H, NH_2), 3.73 (s, 3H, SO_2CH_3), 7.16–7.65 (m, 4H, Ar-H), 8.25 (s, 1*H*, indolyl 2-H), 12.11 (s, 2H, NH_2). EI-MS: *m/z* (%) = 368 (M^+, 13.13). $C_{16}H_{12}N_6O_3S$ (368.37): calcd. C 52.17; H 3.28; N 22.81; found C 52.05; H 3.17; N 22.63.

1,6-diamino-4-(*N*-benzensulfonyl-1*H*-indol-3-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile **4b**; melting point 206–208°C; yield 81%. IR (KBr): υ = 3402 and 3299 (NH_2), 2210 (CN), 1661 (C=O), 1592 (C=C), 1353 and 1162 cm^{-1} (SO_2-N). ^1H NMR (DMSO-*d6*) δ: 2.96 (s, 2H, NH_2), 7.13–8.02 (m, 9H, Ar-H), 8.26 (s, 1*H*, indolyl 2-H), 11.88 (s, 2H, NH_2). EI-MS: *m/z* (%) = 430 (M^+, 0.01). $C_{21}H_{14}N_6O_3S$ (430.44): calcd. C 58.60; H 3.28; N 19.52; found C 58.47; H 3.16; N 19.36.

1,6-diamino-4-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl)-2-oxo-1,2-dihydro-pyridine-3,5-dicarbonitrile **4c**; melting point 159–161°C; yield 93%. IR (KBr): υ = 3419 and 3195 (NH_2), 2210 (CN), 1714 (C=O), 1577 (C=C), 1375 and 1123 (SO_2-N), 747 cm^{-1} (C-Cl). ^1H NMR (DMSO-*d6*) δ: 1.60 (s, 2H, NH_2), 2.98 (s, 2H, NH_2), 7.16–8.17 (m, 8H, Ar-H), 8.32 (s, 1*H*, indolyl 2-H). EI-MS: *m/z* (%) = 464/466 (M^+/M^++2, 0.14/0.04). $C_{21}H_{13}ClN_6O_3S$ (464.88): calcd. C 54.26; H 2.82; N 18.08; found C 54.08; H 2.93; N 17.94.

Synthesis of compounds 5a, 5b, and 5c

A suspension of compound **4a**, **4b**, or **4c** (0.02 mol) in acetic anhydride (10 ml) was heated under reflux for 8–10 h until TLC showed the absence of the starting materials. After cooling, the reaction mixture was poured onto water (20 ml) and the solid formed was filtered off, washed with water, air dried, and crystallized from absolute ethanol.

N-[6-acetylamino-3,5-dicyano-4-(*N*-methanesulfonyl-1*H*-indol-3-yl)-2-oxo-2H-pyridin-1-yl]acetamide **5a**; melting point 245°C; yield 30%. IR (KBr): υ = 3324 and 3206 (NH), 2210 (CN), 1705 and 1689 (C=O), 1612 (C=C), 1386 and 1175 cm^{-1} (SO_2-N). ^1H NMR (DMSO-*d6*) δ: 1.23 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 3.85 (s, 3H, SO_2CH_3), 7.06–7.75 (m, 4H, Ar-H), 8.03 (s, 1*H*, indolyl 2-H), 9.05 (s, 1*H*, NH), 10.21 (s, 1*H*, NH). EI-MS: *m/z* (%) = 452 (M^+, 2.45). $C_{20}H_{16}N_6O_5S$ (452.44): calcd. C 53.09; H 3.56; N 18.57; found C 52.94; H 3.38; N 18.41.

N-[6-acetylamino-3,5-dicyano-4-(*N*-benzenesulfonyl-1*H*-indol-3-yl)-2-oxo-2H-pyridin-1-yl]acetamide **5b**; melting point 134–136°C; yield 44%. IR (KBr): υ = 3284 and 3175 (NH), 2207 (CN), 1710, 1702 and 1645 (C=O), 1564 (C=C), 1365 and 1163 cm^{-1} (SO_2-N). ^1H NMR (DMSO-*d6*) δ: 1.12 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 5.63 (s, 1*H*, NH), 7.11–7.92 (m, 9H, Ar-H), 8.21 (s, 1*H*, indolyl 2-H), 8.65 (s, 1*H*, NH). $C_{25}H_{18}N_6O_5S$ (514.51): calcd. C 58.36; H 3.53; N 16.33; found C 58.21; H 3.36; N 16.19.

N-[6-acetylamino-3,5-dicyano-4-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl)-2-oxo-2H-pyridin-1-yl]acetamide **5c**; melting point 98–100°C; yield 31%. IR (KBr): υ = 3327 and 3219 (NH), 2220 (CN), 1705 and 1672 (C=O), 1595 (C=C), 1387 and 1153 (SO_2-N), 754 cm^{-1} (C-Cl). ^1H NMR (DMSO-*d6*) δ: 2.44 and 2.56 (2s, 6H, $2COCH_3$), 3.63 (s, 1*H*, NH), 7.11–8.52 (m, 9H, Ar-H), 11.90 (s, 1*H*, NH). EI-MS: *m/z* (%) = 548/550 (M^+/M^++2, 3.52/1.14). $C_{25}H_{17}ClN_6O_5S$ (548.96): calcd. C 54.70; H 3.12; N 15.31; found C 54.54; H 3.26; N 15.21.

Synthesis of compounds 6a, 6b, and 6c

A mixture of compound **4a**, **4b**, or **4c** (0.01 mol) and excess of carbon disulfide (10 ml) in an absolute ethanolic potassium hydroxide solution [ethanolic KOH 1 mol/l (20 ml)] was heated under reflux for 12 h. The excess carbon disulfide was evaporated under vacuum and the residue obtained was dissolved in water (20 ml). The reaction mixture was filtered off and the filtrate was acidified with diluted hydrochloric acid (1: 1). The solid that formed was filtered off, washed with water, air dried, and crystallized from dimethylformamide–water.

7-(*N*-methanesulfonyl-1*H*-indol-3-yl)-5-oxo-2-thioxo-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a] pyridine-6,8-dicarbonitrile **6a**; melting point 231–233°C; yield 91%. IR (KBr): υ = 3279 (NH), 2219 (CN), 1725 (C=O), 1568 (C=C), 1384 and 1148 (SO_2-N), 1240 cm^{-1} (C=S). ^1H NMR (DMSO-*d6*) δ: 3.82 (s, 3H, SO_2CH_3), 7.16–8.01 (m, 4H, Ar-H), 8.29 (s, 1*H*, indolyl 2-H), 8.68 (s, 1*H*, NH), 11.87 (s, 1*H*, NH). EI-MS: *m/z* (%) = 410 (M^+, 0.38). $C_{17}H_{10}N_6O_3S_2$ (410.43): calcd. C 49.75; H 2.46; N 20.48; found C 49.56; H 2.31; N 20.52.

7-(*N*-benzenesulfonyl-1*H*-indol-3-yl)-5-oxo-2-thioxo-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a] pyridine-6,8-dicarbonitrile **6b**; melting point 173–175°C; yield 92%. IR (KBr): υ = 3327 (br. NH), 2208 (CN), 1705 (C=O), 1605 (C=C), 1372 and 1175 (SO_2-N), 1245 cm^{-1} (C=S). ^1H NMR (DMSO-*d6*) δ: 7.02–7.75 (m, 9H, Ar-H), 8.25 (s, 1*H*, indolyl 2-H), 11.87 (s, 1*H*, NH), 12.02 (s, 1*H*, NH). EI-MS: *m/z* (%) = 472 (M^+, 0.14). $C_{22}H_{12}N_6O_3S_2$ (472.50): calcd. C 55.92; H 2.56; N 17.79; found C 55.77; H 2.41; N 17.65.

7-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl)-5-oxo-2-thioxo-1,2,3,5-tetrahydro[1,2,4]triazolo[1,5-a] pyridine-6,8-dicarbonitrile **6c**; melting point 179–181°C; yield 88%. IR (KBr): υ = 3312 and 3233 (NH), 2203 (CN), 1643 (C=O), 1572 (C = C), 1373 and 1175 (SO_2-N), 1240 (C=S), 746 cm^{-1} (C-Cl). ^1H NMR (DMSO-d_6) δ: 7.20–8.01 (m, 8H, Ar-H), 8.41 (s, 1*H*, indolyl 2-H), 10.15 (s, 1*H*, NH), 11.86 (s, 1*H*, NH). EI-MS: *m/z* (%) = 506/508 (M^+/M^++2, 0.5/0.16). $C_{22}H_{11}ClN_6O_3S_2$ (506.94): calcd. C 52.12; H 2.19; N 16.58; found C 52.02; H 2.26; N 16.41.

Synthesis of compounds 7a-c and 8a-c

A mixture of compound **4a**, **4b**, or **4c** (0.01 mol) and benzaldehyde or 4-nitro-benzaldehyde (0.01 mol) in absolute ethanol (20 ml) containing glacial acetic acid (0.2 ml) was heated under reflux for 6–10 h. After cooling, the reaction mixture was poured onto ice water (50 ml). The solid that formed was filtered off, washed with water, air dried, and crystallized from benzene.

7-(*N*-methanesulfonyl-1*H*-indol-3-yl)-5-oxo-2-phenyl-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a] pyridine-6,8-dicarbonitrile **7a**; melting point 214°C; yield 83%. IR (KBr): υ = 3286 and 3193 (NH), 2214 (CN), 1655 (C=O), 1523 (C=C), 1371 and 1172 cm^{-1} (SO_2-N). ^1H NMR (DMSO-*d6*) δ: 3.91 (3H, s, SO_2CH_3), 5.66 (1*H*, s, triazolyl 2-H), 6.99–8.37 (10H, m, Ar-H), 8.53 and 8.91 (2H, 2s, 2NH). EI-MS: *m/z* (%) = 456 (M^+, 1.4). $C_{23}H_{16}N_6O_3S$ (456.48): calcd. C 60.52; H 3.53; N 18.41; found C 60.35; H 3.41; N 18.27.

7-(*N*-benzenesulfonyl-1*H*-indol-3-yl)-5-oxo-2-phenyl-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a] pyridine-6,8-dicarbonitrile **7b**; melting point 160–162°C; yield 75%. IR (KBr): υ = 3338 (br. NH), 2212 (CN), 1672 (C=O), 1565 (C=C), 1364 and 1137 cm^{-1} (SO_2-N). ^1H NMR (DMSO-*d6*) δ: 5.31 (s, 1*H*, triazolyl 2-H), 6.55–7.37 (m, 13H, Ar-H), 8.05 (d, 1*H*, indolyl 4-H), 8.40 (s, 1*H*, indolyl 2-H), 9.69 and 9.93 (2s, 2H, 2NH). $C_{28}H_{18}N_6O_3S$ (518.55): calcd. C 64.85; H 3.50; N 16.21; found C 64.67; H 3.35; N 16.04.

7-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl)-5-oxo-2-phenyl-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a]pyridine-6, 8-dicarbonitrile **7c**; melting point 167–169°C; yield 78%. IR (KBr): υ = 3321 and 3164 (NH), 2205 (CN), 1675 (C=O), 1602 (C=C), 1353 and 1197 (SO_2), 743 cm^{-1} (C-Cl). ^1H NMR (DMSO-d_6) δ: 1.13 (s, 1*H*, NH), 5.54 (s, 1*H*, triazolyl 2-H), 7.07–8.04 (m, 13H, Ar-H), 8.22 (s, 1*H*, indolyl 2-H), 9.86 (s, H, NH). EI-MS: *m/z* (%) = 552/554 (M^+/M^++2, 3.44/1.14). $C_{28}H_{17}ClN_6O_3S$ (552.99): calcd. C 60.81; H 3.10; N 15.20; found C 60.66; H 2.93; N 15.01.

7-((*N*-methanesulfonyl)-1*H*-indol-3-yl)-2-(4-nitrophenyl)-5-oxo-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a]pyridine-6,8-dicarbonitrile **8a**; melting point 238–240°C; yield 85%. IR (KBr): υ = 3426 and 3316 (NH), 2203 (CN), 1676 (C=O), 1585 (C=C), 1359 and 1171 cm^{-1} (SO_2-N). ^1H NMR (DMSO-d_6) δ: 4.19 (s, 3H, SO_2CH_3), 5.66 (s, 1*H*, triazolyl 2-H), 7.23–8.09 (m, 9H, Ar-H) 9.58 and 10.04 (2s, 2H, 2NH). EI-MS: *m/z* (%) = 501 (M^+, 3.38). $C_{23}H_{15}N_7O_5S$ (501.47): calcd. C 55.09; H 3.01; N 19.55; found C 54.94; H 3.13; N 19.62.

7-((*N*-benzenesulfonyl)-1*H*-indol-3-yl)-2-(4-nitrophenyl)-5-oxo-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a]pyridine-6,8-dicarbonitrile **8b**; melting point 150–152°C; yield 88%. IR (KBr): υ = 3428 (br. NH), 2214 (CN), 1703 (C=O), 1638 (C=C), 1357 and 1183 cm^{-1} (SO_2-N). ^1H NMR (DMSO-d_6) δ: 5.15 (s, 1*H*, triazolyl 2-H), 7.24–7.94 (13H, m, Ar-H), 8.35 (1*H*, d, indolyl H-2), 8.73 and 10.00 (2s, 2H, 2NH). EI-MS: *m/z* (%) = 563 (M^+, 0.14). $C_{28}H_{17}N_7O_5S$ (563.54): calcd. C 59.68; H 3.04; N 17.40; found C 59.51; H 3.11; N 17.26.

7-((*N*-(4-Chlorobenzenesulfonyl)-1*H*-indol-3-yl)-2-(4-nitrophenyl)-5-oxo-1,2,3,5-tetrahydro[1,2,4] triazolo[1,5-a]pyridine-6,8-dicarbonitrile **8c**; melting point 157–159°C; yield 81%. IR (KBr): υ = 3316 and 3124 (NH), 2204 (CN), 1628 (C=O), 1583 (C=C), 1373 and 1182 (SO_2-N), 755 cm^{-1} (C-Cl). ^1H NMR (DMSO-d_6) δ: 5.60 (s, 1*H*, triazolyl 2-H), 7.19–7.92 (m, 12H, Ar-H), 8.23 (s, 1*H*, indolyl 2-H), 9.88 (s, 1*H*, NH), 12.08 (s, 1*H*, NH). $C_{28}H_{16}ClN_7O_5S$ (597.99): calcd. C 56.24; H 2.70; N 16.40; found C 56.12; H 2.57; N 16.26.

Synthesis of compounds 9a, 9b, and 9c

A mixture of compound **4a**, **4b**, or **4c** (0.01 mol) and 1,2-dibromoethane (1.86 ml, 0.01 mol) in an ethanolic potassium hydroxide solution [ethanolic KOH 1 mol/l (20 ml)] was heated under reflux for 5–6 h. After cooling, the reaction mixture was poured onto

ice water, and then the reaction mixture was filtered off. The filtrate was acidified with diluted hydrochloric acid (1 ml HCl: 1 ml H_2O) and the solid that formed was filtered off, washed with water, air dried, and crystallized from dimethylformamide–water.

8-(*N*-methanesulfonyl-1*H*-indol-3-yl)-6-oxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile **9a**; melting point 202°C; yield 91%. IR (KBr): υ = 3279 and 3160 (NH), 2219 (CN), 1717 (C=O), 1602 (C=C), 1384 and 1148 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 394 (M^+, 0.83). $C_{18}H_{14}N_6O_3S$ (394.41): calcd. C 54.81; H 3.58; N 21.31; found C 54.67; H 3.43; N 21.16.

8-(*N*-benzenesulfonyl-1*H*-indol-3-yl)-6-oxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile **9b**; melting point 300°C; yield 83%. IR (KBr): υ = 3353 (br. NH), 2211 (CN), 1615 (C=O), 1553 (C=C), 1377 and 1128 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 3.25–3.01 (m, 4H, CH_2-CH_2), 7.12–7.76 (m, 9H, Ar-H), 8.03 (s, 1*H*, indolyl 2-H), 8.52 (s, 1*H*, NH), 9.10 (s, 1*H*, NH). $C_{23}H_{16}N_6O_3S$ (456.48): calcd. C 60.52; H 3.53; N 18.41; found C 60.45; H 3.40; N 18.32.

8-[*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl]-6-oxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile **9c**; melting point 158–160°C; yield 86%. IR (KBr): υ = 3369 (NH), 2195 (CN), 1639 (C=O), 1563 (C=C), 1370 and 1175 (SO_2-N), 746 cm^{-1} (C-Cl). ^{1}H NMR (DMSO-*d6*) δ: 1.34 and 1.52 (2s, 2H, 2NH), 2.84 (m, 4H, $2CH_2$), 7.16–8.52 (m, 9H, Ar-H). EI-MS: *m/z* (%) = 490/492 (M^+, 6.35/2.11). $C_{23}H_{15}ClN_6O_3S$ (490.92): calcd. C 56.27; H 3.08; N 17.12; found C 56.12; H 3.14; N 17.04.

Synthesis of compounds 10a, 10b, and 10c

A solution of compound **4a**, **4b**, or **4c** (0.01 mol), chloroacetyl chloride (1.13 ml, 0.01 mol), and triethylamine (0.59 ml, 0.01 mol) in dry 1,4-dioxane (10 ml) was heated under reflux for 15–16 h. The reaction mixture was filtered off while hot and the solvent was removed under vacuum. The residue was triturated with water, filtered off, air dried, and crystallized from absolute ethanol.

8-(*N*-methanesulfonyl-1*H*-indol-3-yl)-2,6-dioxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile **10a**; melting point 97–99°C; yield 88%. IR (KBr): υ = 3280 (NH), 2207 and 2218 (CN), 1712 (C=O), 1567 (C=C), 1348 and 1147 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 2.18 (s, 1*H*, NH), 3.73 (s, 3H, SO_2CH_3), 4.32 (s, 2H, CH_2), 7.13–7.65 (m, 4H, Ar-H), 8.26 (s, 1*H*, indolyl 2-H), 11.88 (s, 1*H*, NH). EI-MS: *m/z* (%) = 408 (M^+, 1.15). $C_{18}H_{12}N_6O_4S$ (408.39): calcd. C 52.94; H 2.96; N 20.58; found C 52.79; H 2.81; N 20.43.

8-(*N*-benzenesulfonyl-1*H*-indol-3-yl)-2,6-dioxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile **10b**; melting point 135–137°C; yield 94%. IR (KBr): υ = 3378 and 3164 (NH), 2215 (CN), 1707 (C=O), 1566 (C=C), 1355 and 1144 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 470 (M^+, 0.68). $C_{23}H_{14}N_6O_4S$ (470.46): calcd. C 58.72; H 3.00; N 17.86; found C 58.57; H 2.91; N 17.72.

8-[*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-yl]-2,6-dioxo-1,3,4,6-tetrahydro-2H-pyrido[1,2-b][1,2,4]triazine-7,9-dicarbonitrile **10c**; melting point 88–90°C; yield 79%. IR (KBr): υ = 3278 and 3176 (NH), 2216 (CN), 1705 and 1699 (C=O), 1565 (C=C), 1386 and 1146 (SO_2-N), 737 cm^{-1} (C-Cl). ^{1}H NMR (DMSO-d_6) δ: 5.46 (s, 2H, CH_2), 5.52 (s, 1*H*, NH), 7.19–7.75 (m, 8H, Ar-H), 8.15 (s, 1*H*, indolyl 2-H), 8.91 (s, 1*H*, NH). $C_{23}H_{13}ClN_6O_4S$ (504.91): calcd. C 54.71; H 2.60; N 16.64; found C 54.56; H 2.45; N 16.49.

Synthesis of compounds 11a, 11b, and 11c

To a solution of compound **2a, 2b,** or **2c** (0.01 mol) in dry ethanol (10 ml) containing triethylamine (0.5 ml), hydrazine hydrate 99% (1 ml, 0.02 mol) was added. The reaction mixture was heated under reflux for 6–8 h. After cooling, the reaction mixture was poured onto ice water (50 ml), and the solid that formed was filtered off, air dried, and crystallized from absolute ethanol.

4-(*N*-methanesulfonyl-1*H*-indol-3-ylmethylene)-4H-pyrazole-3,5-diamine **11a**; melting point 272°C; yield 86%. IR (KBr): υ = 3435 and 3264 (NH_2), 1618 (C=N), 1585 (C=C), 1384 and 1180 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 303 (M^+, 0.28). $C_{13}H_{13}N_5O_2S$ (303.34): calcd. C 51.47; H 4.32; N 23.09; found C 51.32; H 4.17; N 22.97.

4-(*N*-benzenesulfonyl-1*H*-indol-3-ylmethylene)-4H-pyrazole-3,5-diamine **11b**; melting point 134–136°C; yield 81%. IR (KBr): υ = 3391 and 3254 (NH_2), 1618 (C=N), 1521 (C=C), 1380 and 1171 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 3.81 (s, 2H, NH_2), 5.46 (s, 2H, NH_2), 5.52 (s, 1*H*, CH=C), 7.19–7.81 (m, 9H, Ar-H), 8.33 (s, 1*H*, indolyl 2-H). EI-MS: *m/z* (%) = 365 (M^+, 0.19). $C_{18}H_{15}N_5O_2S$ (365.41): calcd. C 59.16; H 4.14; N 19.17; found C 59.04; H 4.02; N 19.06.

4-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-ylmethylene)-4H-pyrazole-3,5-diamine **11c**; melting point 54–56°C; yield 73%. IR (KBr): υ = 3444 and 3265 (NH_2), 1588 (C=N), 1529 (C=C), 1348 and 1178

(SO_2-N), 743 cm^{-1} (C-Cl). EI-MS: *m/z* (%) = 399/401 (M^+/M^++2, 0.01/0.003). $C_{18}H_{14}ClN_5O_2S$ (399.85): calcd. C 54.07; H 3.53; N 17.51; found C 53.95; H 3.37; N 17.38.

Synthesis of compounds 12a, 12b, and 12c

In a mixture of compound **2a, 2b,** or **2c** (0.01 mol), phenylhydrazine (1.08 ml, 0.01 mol) in dry ethanol (10 ml) containing triethylamine (0.5 ml) was heated under reflux for 6–8 h. After cooling, the solid that formed was filtered off, air dried, and crystallized from absolute ethanol.

4-((*N*-methanesulfonyl)-1*H*-indol-3-ylmethylene)-5-imino-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-ylamine **12a**; melting point 130–132°C; yield 77%. IR (KBr): = 3414 (NH_2), 3146 (NH), 1640 (C=N), 1574 (C=C), 1396 and 1150 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 379 (M^+, 0.08). $C_{19}H_{17}N_5O_2S$ (379.44): calcd. C 60.14; H 4.52; N 18.46; found C 60.03; H 4.38; N 18.34.

4-((*N*-benzenesulfonyl)-1*H*-indol-3-ylmethylene)-5-imino-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-ylamine **12b**; melting point 166–168°C; yield 87%. IR (KBr): υ = 3427 (NH_2), 3127 (NH), 1635 (C=N), 1609 (C=C), 1384 and 1163 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 5.47 (s, 1*H*, CH=C), 5.72 (s, 1*H*, NH), 7.15–8.14 (m, 14H, Ar-H), 8.38 (s, 1*H*, indolyl 2-H), 8.52 (s, 2H, NH_2). EI-MS: *m/z* (%) = 441 (M^+, 12.76). $C_{24}H_{19}N_5O_2S$ (441.50): calcd. C 65.29; H 4.34; N 15.86; found C 65.16; H 4.18; N 15.74.

4-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-ylmethylene)-5-imino-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-ylamine **12c**;melting point 158–160°C; yield 81%. IR (KBr): υ = 3450 (NH_2), 3210 (NH), 1639 (C=N), 1575 (C=C), 1383 and 1179 (SO_2-N), 756 cm^{-1} (C-Cl). EI-MS: *m/z* (%) = 475/477 (M^+/M^++2, 0.01/0.003). $C_{24}H_{18}ClN_5O_2S$ (475.95): calcd. C 60.56; H 3.81; N 14.71; found C 60.41; H 3.66; N 14.56.

Synthesis of compounds 13a–c and 14a–c

A mixture of compound **2a**, **2b**, or **2c** (0.01 mol) and urea or thiourea (0.01 mol) in dry ethanol (10 ml) containing triethylamine (0.5 ml) was heated under reflux for 8–10 h. After cooling, the reaction mixture was poured onto ice water (50 ml) and the solid that formed was filtered off, air dried, and crystallized from absolute ethanol to yield **13a–c** or **14a–c**, respectively.

4,6-diamino-5-(*N*-methanesulfonyl-1*H*-indol-3-ylmethylene)-5H-pyrimidin-2-one **13a**; melting point 114–116°C; yield 77%. IR (KBr): υ = 3212 and 3172 (NH_2), 1706 (C=O), 1644 (C=N), 1584 (C=C), 1387 and 1139 (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 3.73 (s, 3H, SO_2CH_3), 5.57 (s, 1*H*, CH=C), 7.06–7.48 (m, 4H, Ar-H), 8.13 (s, 1*H*, indolyl 2-H), 9.90 (s, 2H, NH_2), 12.11 (s, 2H, NH_2). EI-MS: *m/z* (%) = 331(M^+, 0.25). $C_{14}H_{13}N_5O_3S$ (331.35): calcd. C 50.75; H 3.95; N 21.14; found C 50.59; H 3.77; N 21.05.

4,6-diamino-5-((*N*-benzenesulfonyl)-1*H*-indol-3-ylmethylene)-5H-pyrimidin-2-one **13b**; m.p. 110–112°C; yield 71%. IR (KBr): υ = 3450 and 3272 (NH_2), 1703 (C=O), 1639 (C=N), 1575 (C=C), 1383 and 1179 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 393 (M^+, 0.02). $C_{19}H_{15}N_5O_3S$ (393.42): calcd. C 58.01; H 3.84; N 17.80; found C 58.12; H 3.71; N 17.64.

4,6-diamino-5-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-ylmethylene)-5H-pyrimidin-2-one **13c**; melting point 150–152°C; yield 69%. IR (KBr): υ = 3427 and 3228 (NH_2), 1712 (C=O), 1634 (C=N), 1609 (C=C), 1384 and 1163 (SO_2-N), 744 cm^{-1} (C-Cl). ^{1}H NMR (DMSO-*d6*) δ: 2.04 (s, 2H, NH_2), 2.47 (s, 2H, NH_2), 5.57 (s, 1*H*, CH=C), 7.17–7.78 (m, 9H, Ar-H), 8.15 (s, 1*H*, indolyl 2-H). $C_{19}H_{14}ClN_5O_3S$ (427.86): calcd. C 53.34; H 3.30; N 16.37; found C 53.17; H 3.21; N 16.23.

4,6-diamino-5-(*N*-(4-methanesulfonyl)-1*H*-indol-3-ylmethylene)-5H-pyrimidine -2-thione **14a**; melting point 125–127°C; yield 67%. IR (KBr): υ = 3401 and 3212 (NH_2), 1623 (C=N), 1526 (C=C), 1379 and 1130 (SO_2-N), 1244 cm^{-1} (C=S). ^{1}H NMR (DMSO-*d6*) δ: 2.46 (s, 2H, NH_2), 4.14 (s, 3H, SO_2CH_3), 5.23 (s, 2H, NH_2), 5.71 (s, 1*H*, CH=C), 7.07–7.76 (m, 4H, Ar-H), 8.18 (s, 1*H*, indolyl 2-H). EI-MS: *m/z* (%) = 347 (M^+, 0.02). $C_{14}H_{13}N_5O_2S_2$ (347.42): calcd. C 48.40; H 3.77; N 20.16; found C 48.25; H 3.63; N 20.03.

4,6-diamino-5-(*N*-benzenesulfonyl-1*H*-indol-3-ylmethylene)-5H-pyrimidine-2-thione **14b**; melting point 130–132°C; yield 65%. IR (KBr): υ = 3396 and 3255 (NH_2), 1618 (C=N), 1523 (C=C), 1386 and 1175 (SO_2), 1240 cm^{-1} (C=S). EI-MS: *m/z* (%) = 409 (M^+, 55.55). $C_{19}H_{15}N_5O_2S_2$ (409.48): calcd. C 55.73; H 3.69; N 17.10; found C 55.55; H 3.54; N 17.02.

4,6-diamino-5-(*N*-(4-chlorobenzenesulfonyl)-1*H*-indol-3-ylmethylene)-5H-pyrimidine-2-thione **14c**; melting point 109–111°C; yield 62%. IR (KBr): υ = 3400 and 3228 (NH_2), 1618 (C=N), 1590 (C=C), 1383 and 1179 (SO_2-N), 1243 (C=S), 755 cm^{-1} (C-Cl). EI-MS: *m/z* (%) = 443/445 (M^+/M^++2, 7/2). $C_{19}H_{14}ClN_5O_2S_2$ (443.93): calcd. C 51.41; H 3.18; N 15.78; found C 51.29; H 3.04; N 15.66.

Synthesis of compounds 15a, 15b, and 15c

A mixture of compound **2a**, **2b**, or **2c** (0.01 mol), guanidine hydrochloride (0.96 g, 0.01 mol), and triethylamine (1 ml) in dry ethanol (15 ml) was heated under reflux for 2–3 h. After cooling, the solid that formed was filtered off, air dried, and crystallized from absolute ethanol.

2-imino-5-(*N*-methanesulfonyl-1*H*-indol-3-ylmethylene)-2,5-dihydro-pyrimidine-4,6-diamine **15a**; m.p. 98–100˚C; yield 66%. IR (KBr): υ = 3403 and 3249 (NH_2), 3173 (NH), 1634 (C=N), 1526 (C=C), 1385 and 1180 cm^{-1} (SO_2-N). EI-MS: *m/z* (%) = 330 (M^+, 1.11). $C_{14}H_{14}N_6O_2S$ (330.36): calcd. C 50.90; H 4.27 N 25.44; found C 50.78; H 4.14; N 25.32.

2-Imino-5-(N-benzenesulfonyl-1*H*-indol-3-ylmethylene)-2,5-dihydropyrimidine-4,6-diamine **15b**; melting point 140–142˚C; yield 61%. IR (KBr): υ = 3443, 3378 and 3273 (NH_2), 3121 (NH), 1624 (C=N), 1569 (C=C), 1350 and 1176 cm^{-1} (SO_2-N). ^{1}H NMR (DMSO-*d6*) δ: 1.60 (s, 2H, NH_2), 2.98 (s, 2H, NH_2), 5.46 (s, 1*H*, CH=C), 6.52 (s, 1*H*, NH), 7.16–7.82 (m, 9H, Ar-H), 8.21 (s, 1*H*, indolyl 2-H). EI-MS: *m/z* (%) = 392 (M^+, 0.08). $C_{19}H_{16}N_6O_2S$ (392.43): calcd. C 58.15; H 4.11; N 21.42; found C 58.07; H 4.01; N 21.35.

2-imino-5-(*N*-(4-chlorobenzenesulfonyl-1*H*-indol-3-ylmethylene)-2,5-dihydropyrimidine-4,6-diamine **15c**; melting point 90–92˚C; yield 54%. IR (KBr): υ = 3428 and 3327 (NH_2) 3195 (NH), 1624 (C=N), 1554 (C=C), 1336 and 1150 (SO_2-N), 751 cm^{-1} (C-Cl). ^{1}H NMR (DMSO-*d6*) δ: 1.87 (s, 2H, NH_2), 5.47 (s, 1*H*, CH=C), 5.72 (s, 1*H*, NH), 7.15–7.82 (m, 8H, Ar-H), 8.18 (s, 1*H*, indolyl 2-H), 8.52 (s, 2H, NH_2). $C_{19}H_{15}ClN_6O_2S$ (426.88): calcd. C 53.46; H 3.54; N 19.69; found C 53.31; H 3.38; N 19.52.

Biological assay

Cell propagation and maintenance

Hepatocellular carcinoma HepG2 cells were purchased from the holding company for biological products and vaccines (VACSERA, Agouza, Giza, Egypt) and maintained under the proper conditions. The cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 100 IU/ml penicillin G sodium, 100 lU/ml streptomycin sulfate, 1% L-glutamine, and 10% fetal bovine serum at 37˚C in a humidified incubator with 5% CO_2. The cells were harvested after trypsinization (0.025% trypsin and 0.02% EDTA) and washed twice with Dulbecco's PBS (Bio-Whittaker, Lonza, Verviers, Belgium). When the cell density reached ~80%, cells were split for further culture. The experiments were conducted when the cells were in the logarithmic growth phase.

Cytotoxicity assay

Cell viability was measured using a neutral red uptake assay [26]. The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The cells were incubated with various concentrations of the test compounds (25, 125, 250, 500, and 1000 μmol/l) for 48 h at a cell density of 10^4 cells/well of a 96-well plate. A neutral red working solution (0.4 μg/ml) (Sigma-Aldrich) was incubated overnight at 37˚C in the same manner as the treated cells.

In each well of the incubated cells, culture media were removed and neutral red medium (100 μl) was added, and then incubated for 2 h to allow for vital dye incorporation into living cells. The neutral red media were removed and rinsed rapidly with Dulbecco's PBS buffer (150 μl). Dye was extracted from the cells by adding extraction buffer [150 μl, 1% acetic acid : 50% ethanol (96%): 49% deionized H_2O], followed by rapid agitation for at least 10 min on a micrometer plate shaker. The extract neutral red color intensity was measured at 530 and 645 nm as excitation and emission wavelengths in a micro-titer plate reader spectrophotometer (Sorin, Biomedica S.p.A., Milan, Italy). Using the relation between the concentrations used and the neutral red intensity value, the IC_{50} of the tested compounds was calculated. For the untreated cells (negative control), medium was added instead of the test compounds. A positive control Adrinamycin (doxorubicin) (Mr = 579.9) was used as a cytotoxic natural agent yielding 100% inhibition. DMSO was the vehicle used for dissolution of the tested compound and its final concentration on the cells was less than 0.2%. All tests and analyses were carried out in triplicate and the results were averaged.

Gene expression analysis

HepG2 cells were seeded (4 × 10^4 cells/well) and incubated with different treatments at 37˚C for 48 h. The total RNA was isolated using PeqGold Trifast (Biotechnologie GmbH, Erwin-Rentschler-Strasse 21 Laupheim, 88471, German) according to the manufacturer's instructions. The primer sequence for Beta-actin gene is as follows: forward, CCTTCCTGGGCATGGAGTCCT; reverse, GGAGCAATGATCTTGA TCTTC. Primer sequence for *BCL-2* gene is: forward,

CCTGGTGGACAACATCGCC; reverse, AATCAAACAGAGGCCG CATGC. Qiagen on step RT PCR kit (Qiagen Inc., Valencia, California, USA) was used for RNA reverse transcription and subsequent amplification. PCR reaction was performed separately for Beta-actin and *BCl-2* by adding 2 μg RNA to the PCR mixture and making the reaction volume up to 50 μl. The PCR mixture included 2 mmol/l Tris-HCl, 10 mmol/l KCl, NH_2SO_4, 1.25 m,ol/l $MgCl_2$, and 0.1 mmol/l dithiothreitol, pH 8.7, 0.4 mmol/l dNTPs mixture, Qiagen one step RT PCR enzyme, Mix (Omniscript TM reverse transcriptase, Sensiscript TM reverse transcriptase and Hot star TaqR DNA polymerase), and 0.6 μmol/l of each specific primer. The reaction mixture was subjected to reverse transcription at 50°C for 30 min and then to 35 cycles of PCR amplification (BioRad-T100 thermal cycler; BioRad, Hercules, California, USA) as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were separated on a 1.5% agarose gel and visualized using a gel documentation system. The genes' expressions were semiquantified using LabImage analysis (Labmage version 2.7.0, Kapelan Bio-Imagin GmbH, Leipzig, Germany) software.

Statistical analysis

Data were analyzed using version 13 of a computer-based statistical package (SPSS Inc., Chicago, IL, USA). Results are expressed as means ± SD of three independent experiments. Statistical significance of difference was determined using analysis of variance (one-way analysis of variance). Further statistical analysis for post hoc comparisons was carried out using the LSD test. A level of *P* more than 0.05 was considered to be statistically significant.

Molecular docking studies

Docking studies of the most proapoptotic active compounds were carried out using the Molecular Operating Environment (MOE) 2008.10 release of Chemical Computing Group (Montereal, Canada; *http://www.chemcomp.com*). The program operated on an Intel core i3-32100 CPU@3.10 GHz 3.09 GHz processor, 3.41 GB of RAM, Microsoft Windows XP (Microsoft Corporation, 1 Microsoft Way, Redmond, WA, 98052, United States). The protein crystal structure of CHIMAERIC *BCL2*-XL in complex with 1-(2-{[(3S)-3-(aminomethyl)-3,4-dihydroisoquinolin-2(1*H*)-yl]carbonyl}phenyl)-4-chloro-5-methyl-*N,N*-diphenyl-1*H*-pyrazole-3-carboxamide was downloaded from *http://www.rcsb.org/pdb* (PDB ID: 2W3L) as selective *BCL-2* inhibitors [27].

The protein crystal structure was prepared for docking by removing water molecules, adding and removing polar hydrogen atoms, and then isolating the active pocket. The active pocket was considered to be the site where the cocrystalline ligand, namely, tetrahydroisoquinoline amide complexes with the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L). The active pocket consisted of 12 amino acid residues such as Leu96, Ala108, Phe112, Phe63, Tyr67, Met74, Phe71, Asp70, Val92, Glu95, Arg108, and Gly104.

The cocrystalline ligand was redocked in the active pocket to insure that the docking method was efficient, and the active pocket was saved as a MOE file to be used for docking simulation of the selected compounds (ligands).

The structures of the selected compounds (ligands) for docking were drawn in ChemDraw Ultra 10.0 (Cheminformatics Software company based in Cambridge, Massachusetts, USA) and saved as mol. Before the docking, preparation steps had to be taken as follows:

(a) Conversion of the 2D structure of ligands into their 3D form;
(b) Addition and removal of polar hydrogen atoms;
(c) Energy minimization using the MMFF94x forcefield until a root-mean-square deviation of the atomic position gradient of 0.01 kcal/mol/Å was reached and then it was saved as moe.

MMFF94x was reported as the efficient force field for minimization of ligand–protein complexes [28].

The docking algorithm was performed by MOE-DOCK default. It uses a flexible rigid technique to place the molecule within the cavity. All rotatable bonds of ligands are allowed to undergo free rotation for placement in a rigid receptor-binding site.

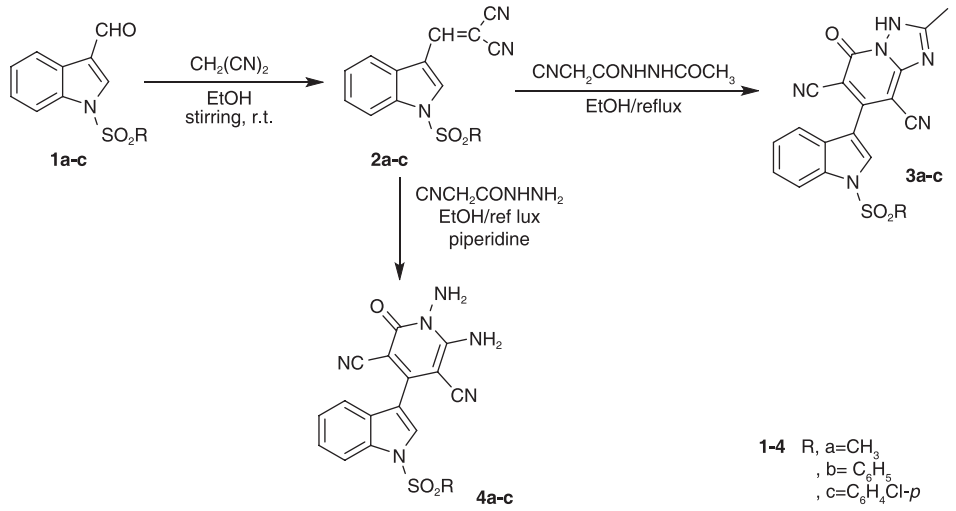
The docking scores were expressed in negative energy terms; the lower the binding free energy, the better the binding affinity [29].

Results and discussion

Chemistry

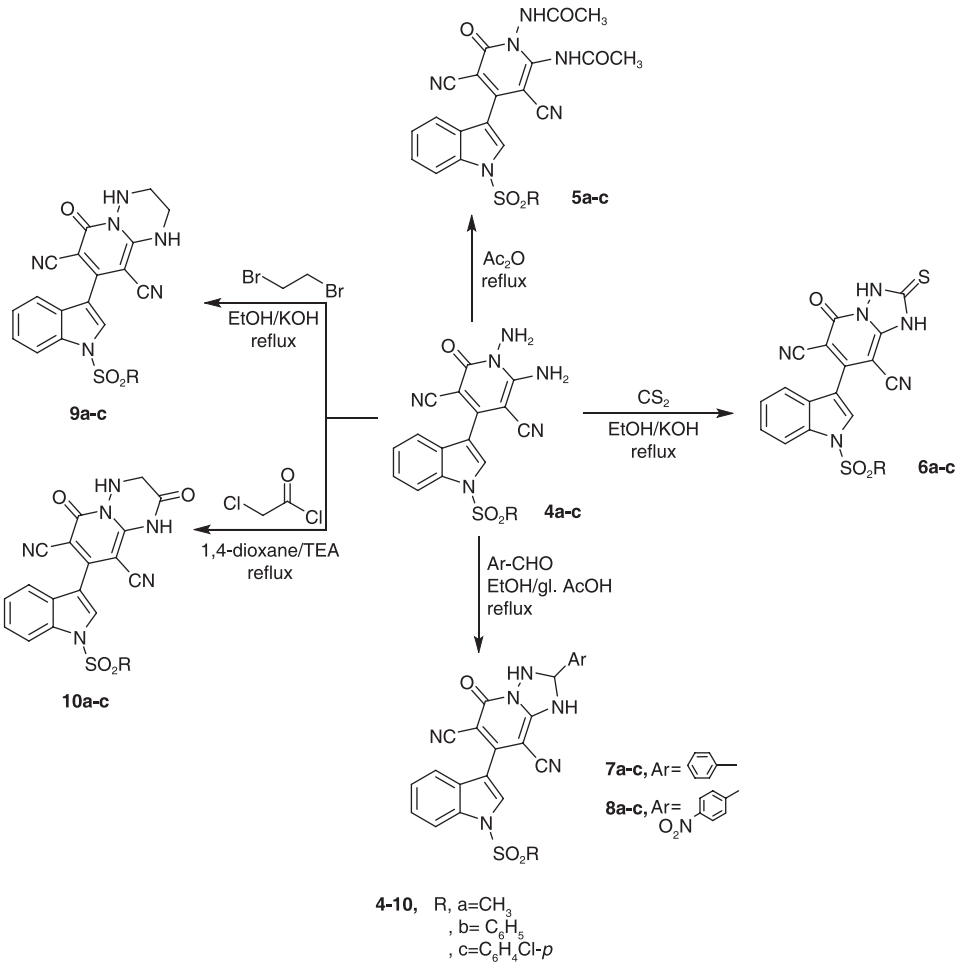
The synthetic routes of the target compounds are outlined in Schemes 1–3. The starting 2-(*N*-substituted sulfonyl-1*H*-indol-3-yl)methylene)malononitriles **2a–c** was prepared by a base-catalyzed reaction of *N*-methyl, *N*-phenyl and *N*-(*p*-chlorobenzene) sulfonyl-3-indolyl carboxaldehydes (**1a–c**) with malononitrile in absolute ethanol under stirring at room temperature (Scheme 1). Compound **2b** has

Scheme 1



Synthesis of compounds **2a–c**, **3a–c**, and **4a–c**.

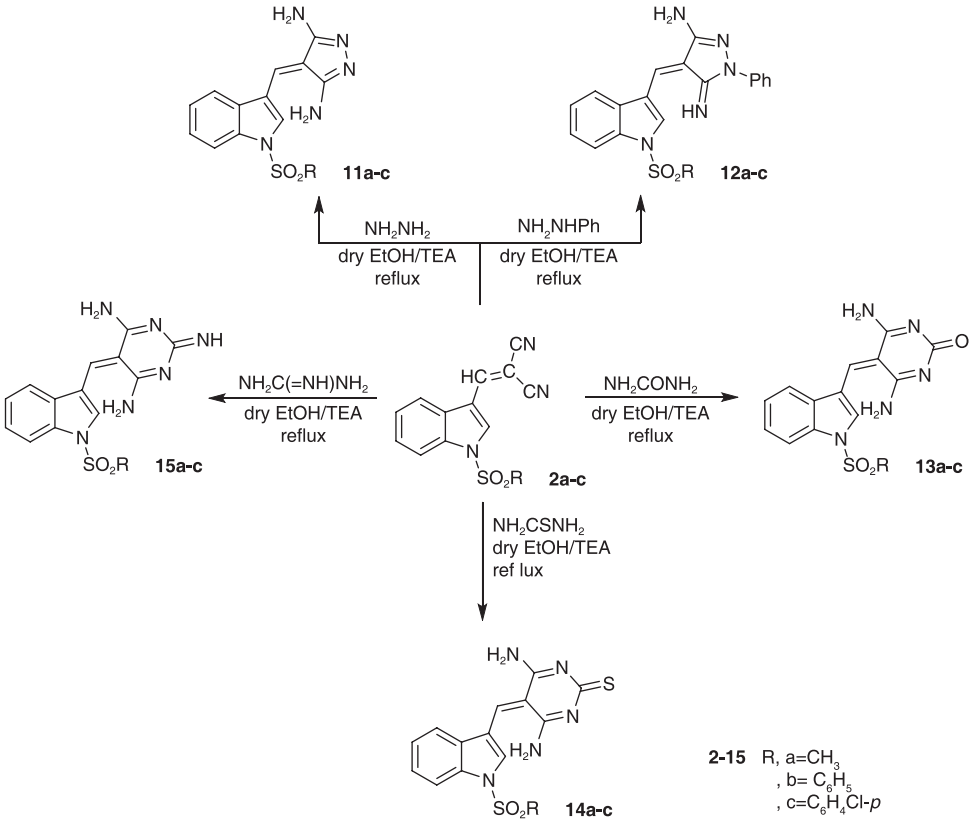
Scheme 2



Synthesis of pyridine diacetamides **5a–c**, triazolo[1, 5-a]pyridines **6a–c**, **7a–c**, **8a–c** and pyrido[1, 2, 4]triazines derivatives **9a–c, 10a–c**.

been reported previously [25], whereas compounds **2a** and 2c are new and their structure were confirmed on the basis of their correct elemental analyses, and IR and ^{1}H NMR spectra. IR spectra of **2a** and **2c** showed characteristic absorption bands at 2207 and 2195 cm^{-1} because of the CN group. Their ^{1}H NMR showed

Scheme 3



Synthesis of pyrazoles **11a–c**, **12a–c** and pyrimidines **13a–c–15a–c**.

singlet signals at δ 7.84 and 7.62 ppm because of CH=C protons.

Cyclocondensation of **2a–c** with 2'-acetyl-2-cyanoacetohydrazide in absolute ethanol containing triethylamine as a catalyst led to the formation of fused 7-(*N*-substituted sulfonyl-1*H*-indol-3-yl)-2-methyl-5-oxo-3,5-dihydro-[1,2,4]triazolo[1,5-*a*]pyridine-6,8-dicarbonitriles **3a–c** (Scheme 1). IR spectra of each **3a, 3b**, and **3c** showed additional absorption bands at ~1703–1730 cm^{-1} characteristic of the C=O group. ^1H NMR of 3a and 3c lack the presence of CH=C and showed new singlet signals at δ 2.17 and 1.19 ppm, respectively, for (3H, CH_3), in addition to D_2O exchangeable singlet signals at δ 8.12 and 8.78 ppm, respectively, for NH protons.

However, the reaction of compounds **2a–c** with 2-cyanoacetic acid hydrazide in refluxing ethanolic piperidine yielded the corresponding 1,6-diamino-4-(*N*-substituted sulfonyl-1*H*-indol-3-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitriles **4a–c** (Scheme 1). ^1H NMR spectra of **4a–c** showed D_2O exchangeable singlet signals at δ~1.14–2.96 and 2.98–12.11 ppm because of C-NH_2 and N-NH_2 protons, respectively.

Acetylation of 1,6-diaminopyridines **4a–c** using acetic anhydride led to the formation of the corresponding diacetamides, namely, *N*-(6-acetylamino-3,5-dicyano-4-(*N*-substituted sulfonyl-1*H*-indol-3-yl)-2-oxo-2*H*-pyridin-1-yl)acetamides **5a–c** (Scheme 2).

Moreover, compounds **4a–c** were used as starting materials for build-up of the fused heterocyclic system through a ring closure reaction of their α,β-bifunctional amino groups. Cyclization of **4a–c** with excess carbon disulfide in an ethanolic potassium hydroxide solution yielded the fused **7**-(*N*-substituted sulfonyl-1*H*-indol-3-yl)-5-oxo-2-thioxo-1,2,3,5-tetrahydro[1,2,4]triazolo[1,5-α]pyridine-6,8-dicarbonitriles **6a–c** (Scheme 2).

Furthermore, the reaction of compounds **4a–c** with aromatic aldehydes, namely, benzaldehyde and/or 4-nitrobenzaldehyde under reflux in absolute ethanol containing a catalytic amount of glacial acetic acid yielded the fused triazolo[1,5-α]pyridine derivatives **7a–c** and **8a–c** (Scheme 2). ^1H NMR spectra of each **7a–c** and **8a–c** showed singlet signals at δ 5.66, 5.31, 5.54, 5.66, 5.15, and 5.60 ppm, respectively, attributable to (1*H*, triazolyl 2-H), in addition to D_2O exchangeable singlet signals at δ ~1.13–12.08 ppm because of NH protons besides the aromatic protons.

However, the reaction of compounds **4a–c** with 1,2-dibromoethane under reflux in an ethanolic potassium hydroxide solution yielded 8-(*N*-substituted sulfonyl-1*H*-indol-3-yl)-6-oxo-1,3,4,6-tetrahydro-2*H*-pyrido [1,2-*b*][1,2,4]triazine-7,9-dicarbonitrile **9a–c** (Scheme 2). Reaction of compounds **4a–c** with choloroacetyl chloride under reflux in dry 1,4-dioxane yielded 8-(*N*-substituted sulfonyl-1*H*-indol-3-yl)-2,6-dioxo-1,3,4,6-tetrahydro-2*H*-pyrido[1,2-*b*][1,2,4] triazine-7,9-dicarbonitriles **10a–c** (Scheme 2).

Various arylidene malononitriles have been used as intermediates for the synthesis of pyrazole, pyrimidine, and pyridine derivatives [30]. In the present study, the reaction of compounds **2a–c** with hydrazine hydrate and phenyl hydrazine in dry ethanol containing a catalytic amount of triethylamine led to the formation of the new pyrazole derivatives **11a–c** and **12a–c** (Scheme 3). The reaction of compounds 2a–c with urea and/or thiourea in dry ethanol containing triethylamine yielded the corresponding pyrimidin-2-ones **13a–c** and pyrimidine-2-thiones **14a–c**, respectively (Scheme 3).

Finally, cyclization of compounds **2a–c** with guanidine hydrochloride under reflux in dry ethanol containing triethylamine yielded 2-imino-5-(*N*-substituted sulfonyl-1*H*-indol-3-ylmethylene)-2,5-dihydropyrimidine-4,6-diamines **15a–c** (Scheme 3).

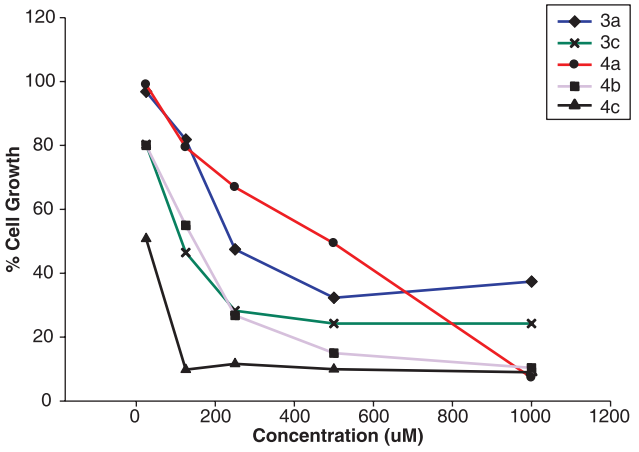
Biological assay

Cytotoxic activity

The newly synthesized compounds were investigated individually as anticancer agents against the human hepatocellular carcinoma cell line (HepG2) at concentrations of 25, 125, 250, 500, and 1000 mmol/l. The inhibition of proliferation of the HepG2 cell line was determined using a neutral red assay, which is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Doxorubicin was used as a reference drug (IC_{50}, 3.4 μmol/l). The use of DMSO as a solvent had an insignificant effect on the viability of HepG2 cells when treated for 48 h. From the data obtained (Table 1, Figs 1–3), the most active compounds against the HepG2 cancer cell line were in the descending order of **10b>4c>10a>10c**. The existence of 1,3,4,6-tetrahydro-2*H*-pyrido[1,2-*b*] [1,2,4]triazine at position-3 of indole as in compounds **10a**, **10b** ,and **10c** showed growth inhibition with IC_{50} values of 35, 24, and 40 μmol/l, respectively, compared with the control cell. However, the existence of chlorine atom at the *para* position of the benzenesulfonyl moiety at the *N*-position of indole with 1,6-diamino-1,2-dihydropyridine at position-3 of indole as in **4c** showed growth inhibition with an IC_{50} of 30 μmol/l.

Furthermore, compounds **3c**, **6c**, **9c**, **4b**, and **5c** showed moderate to slight growth inhibition with IC_{50} values

Figure 1



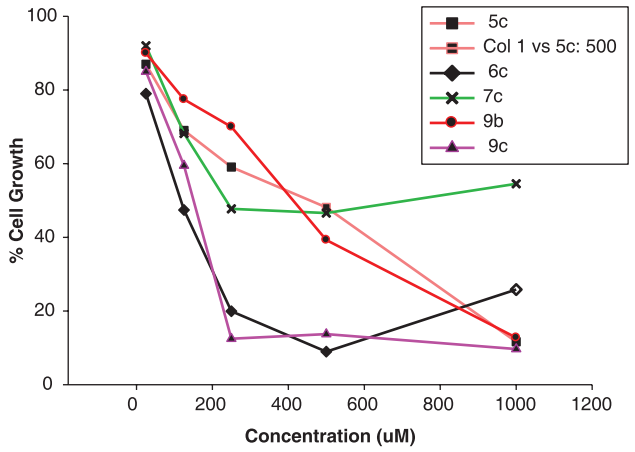
Effects of test compounds **3a, 3c, 4a, 4b** and **4c** on HepG2 cell line at 48 h incubation time.

Table 1 ***In-vitro*** **cytotoxic activity of the newly synthesized compounds on the HepG2 cancer cell line**

Compound number	IC_{50} (μmol/l)	Compound number	IC_{50} (μmol/l)	Compound number	IC_{50} (μmol/l)
3a	240	**7b**	>1000	**11c**	800
3b	>1000	**7c**	220	**12a**	220
3c	110	**8a**	>1000	**12b**	>1000
4a	480	**8b**	>1000	**12c**	>1000
4b	150	**8c**	>1000	**13a**	>1000
4c	30	**9a**	>1000	**13b**	>1000
5a	>1000	**9b**	390	**13c**	320
5b	760	**9c**	147	**14a**	>1000
5c	180	**10a**	35	**14b**	>1000
6a	550	**10b**	24	**14c**	640
6b	560	**10c**	40	**15a**	220
6c	120	**11a**	>1000	**15b**	800
7a	587	**11b**	>1000	**15c**	460
Doxorubicin	3.4	Doxorubicin	3.4	Doxorubicin	3.4

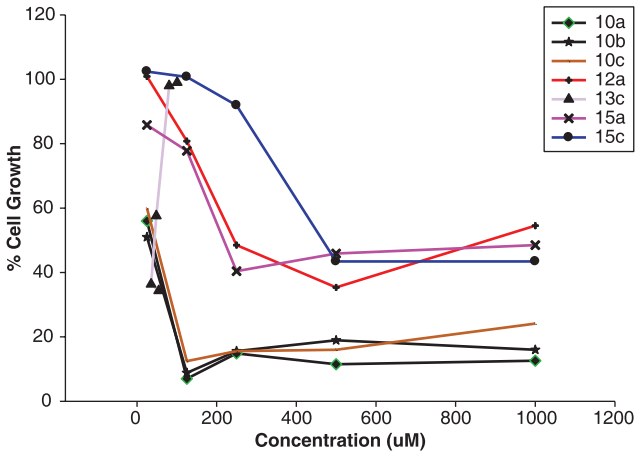
IC_{50}, concentration required to inhibit cell viability by 50%; >1000; inactive.

Figure 2



Effects of test compounds **5c**, **6c**, **7c**, **9b**, and **9c** on the HepG2 cell line at 48 h incubation time.

Figure 3



Effects of test compounds **10a**, **10b**, **10c**, **12a**, **13c**, **15a**, and **15c** on the HepG2 cell line at 48 h incubation time.

of 110,120, 147, 150, and 180 mmol/l, respectively (Table 1, Figs 1–3). However, the rest of the compounds showed no activity against the HepG2 cancer cell line compared with control cells (Table 1). The activities of compounds **3c**, **6c**, **9c**, and **5c** seem to be related to the presence of chlorine atoms at the *para* position of the benzenesulfonyl moiety.

BCL-2 gene expression patterns

The *BCL*-2 gene binds to the outer mitochondrial membrane and increases cell survival by acting as an anti-apoptotic factor, rather than by promoting cell proliferation. It has also been identified as an oncogene that prevents apoptosis in tumor cells [2]. There are a number of theories on how the *Bcl*-2 gene family exerts its proapoptotic or anti-apoptotic effect. An important one states that this is achieved by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix Ca^{++}, pH, and voltage. It is also believed that some *Bcl*-2 family proteins can induce (proapoptotic members) or inhibit (anti-apoptotic members) the release of cytochrome c into the cytosol, which, once there, activates caspase-9 and caspase-3, leading to apoptosis. Strong evidence suggests an earlier implication of the mitochondrial apoptosis-induced channel pore on the outer membrane [31,32]. On the basis of these findings, we investigated the effect of the proposed treatments on *BCL*-2 gene expression.

The newly synthesized compounds **3c**, **4b**, **4c**, **5c**, **6c**, **9c**, **10a**, **10b**, and **10c**, which showed IC_{50} values ranging from 24 to 180 µmol/l, were chosen to study their proapoptotic effects. *BCL*-2 gene expression levels were determined by calculation of the ratio of its expression level to that of β-actin by a semi-quantitative analysis. The result showed that compounds **4c**, **5c**, **9c**, **10a**, **10b**, and **10c** significantly inhibited the expression levels of the *BCL*-2 gene (Fig. 4). Our finding is similar to that of Lin *et al.* [33], who reported that novel 6-acetyl-9-(3,4,5-trimetho-xybenzyl)-9H-pyrido[2,3-*b*]indole induces mitotic arrest and apoptosis in human COLO 205 cells. This anticancer effect was attributed to the ability of the indole derivative to disrupt microtubule assembly and induce G2/M arrest, polyploidy, and apoptosis by mitochondrial pathways in COLO 205 cells. In addition, it reduced the levels of procaspase-3, procaspase-9, *BCL*-xL, and *BCL*-2 gene [33].

Molecular docking studies

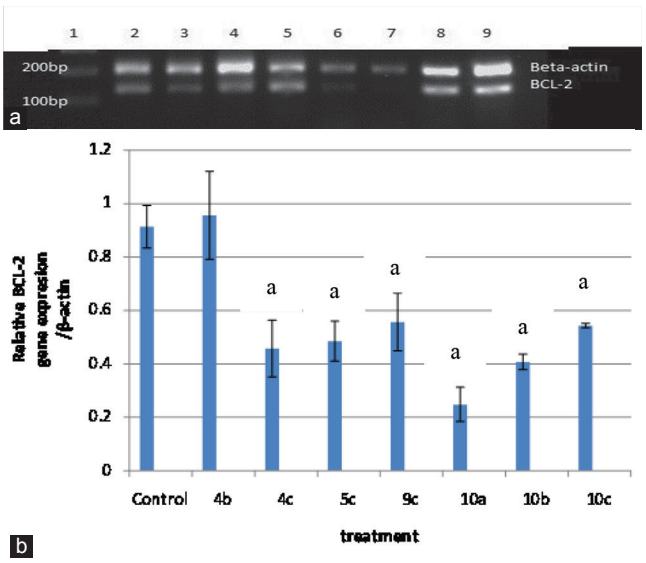
The result of the proapoptotic effects of the tested compounds led us to carry out molecular docking studies to understand the ligand–protein interactions in detail. Compounds **4b**, **4c**, **5c**, **9c**, **10a**, **10b**, and **10c** were docked against the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L) using MOE 2008.10 program. From the data obtained (Table 2, Figs 5–7), it was found that compounds **5c** and **9c** show good fitting inside the pockets site of the protein molecular surface and had a minimum binding energy of -20.29 and -18.98 kJ/mol, respectively, in comparison with the cocrystallized ligand 1-(2-{[(3S)-3-(aminomethyl)-3,4-dihydro-isoquinolin-2(1*H*)yl]carbonyl}phenyl)-4-chloro-5-methyl-*N*,*N*-diphenyl-1*H*-pyrazole-3-carboxamide, which has a binding energy of –16.28 kJ/mol, an root-mean-square deviation value of 1.95, and formed only one hydrogen bond at distance 2.30 Å (Table 2, Fig. 5a and b).

Compound **5c**, in which *N*,*N*′-diacetamido-2-oxopyridine incorporated into an indole ring, forms an H-bond with the amino acid residues of the pocket site, C=O of the acetamido group, with a C=O of Tyr67 at

a distance of 2.76 Å (Fig. 6a and b). This compound has the best docking score with a minimum binding energy of (−20.29 kJ/mol), which correlates with the result of the pro-apoptotic effect.

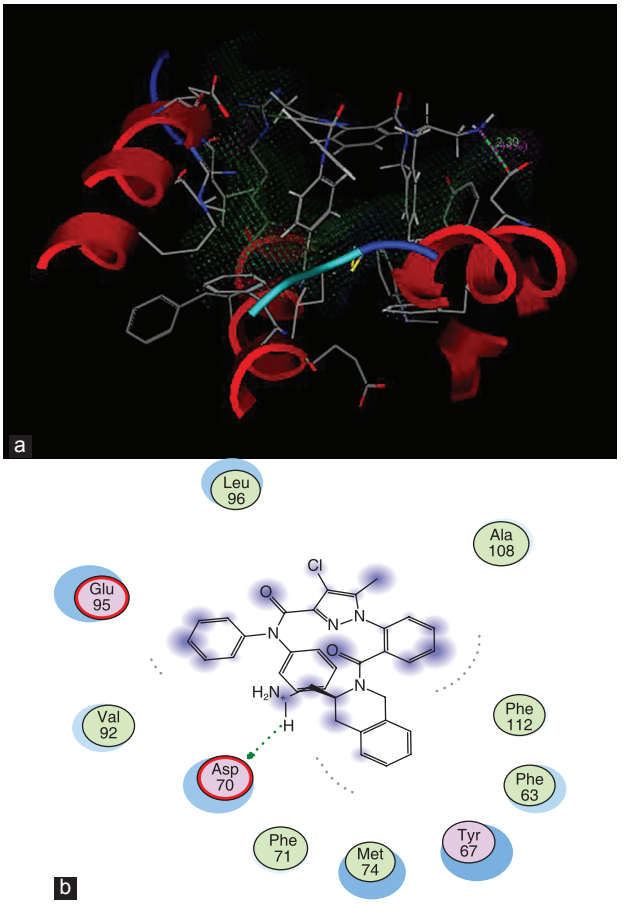
Replacement of the acetamido group by the triazine moiety as in compound **9c** maintained the good fit within the pocket site of the protein molecular surface, with one H-bond formed between C=O of pyridine and NH of Arg105 at a distance of 2.56 Å, in addition to an arene–arene link between the phenyl ring of indole and the phenyl ring of Tyr67 (Fig. 7a and b), but with a binding energy of (−18.98 kJ/mol) higher than the cocrystal ligand and less than compound **5c**.

Figure 4



(a) RT–PCR product of the *BCL*-2 gene and b-actin genes expressed in the hepatocellular carcinoma cell line (HepG2). Lane 1 represents the DNA marker; lanes 2–9 represent compounds **4b**, **4c**, **5c**, **9c**, **10a**, **10b**, **10c**, and control, respectively. (b) Expression of the *BCL*-2 gene in the hepatocellular carcinoma cell line (HepG2). The gene expression was estimated as the ratio between the intensity of the *BCL*-2 gene and the β-actin gene. Data are expressed as mean ± SE. 'a' represents a significant difference compared with the control ($P < 0.05$).

Figure 5

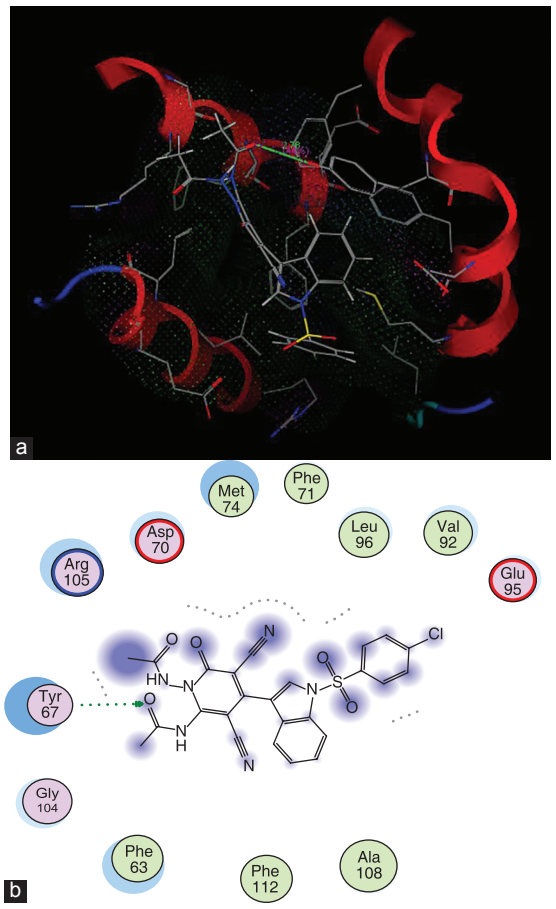


(a) Docked conformation alignment of cocrystallized ligand (tetrahydroisoquinoline amide) in the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L)-binding site. (b) Simplified structure showing an interaction between tetrahydroisoquinoline amide and the amino acid residues in the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L) active site.

Table 2 Docking results of the most active compounds that docked with the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L)

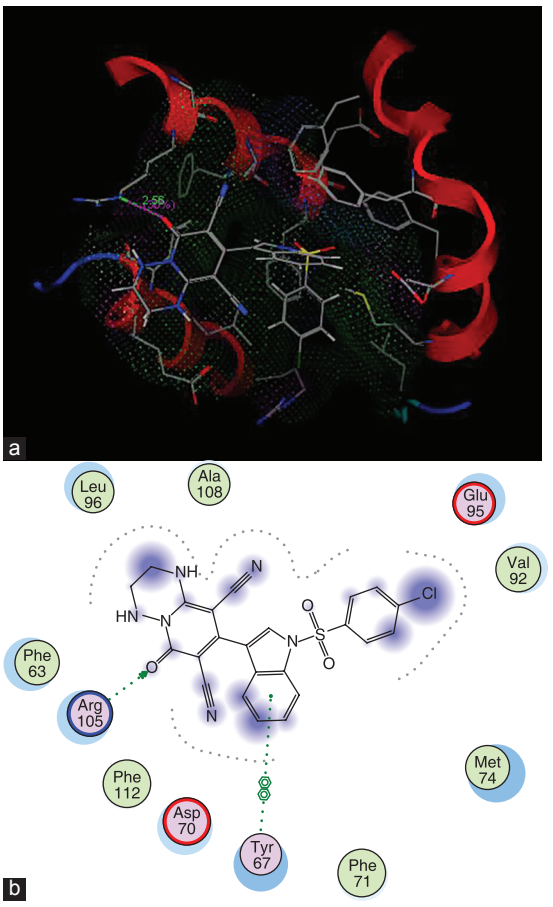
Compound number	Binding energy (kJ/mol)	Main atoms from the compounds	Main residue from 2W3L	Distance (Å)
Cocrystallized ligand	−16.28	NH	C=O of Asp70	2.30
4b	−11.63	N-NH_2	CO of Tyr67	2.00
		CN	NH of Arg105	3.32
4c	−15.89	N-NH_2	CO of Leu96	3.10
5c	−20.29	C=O	C=O of Try67	2.76
9c	−18.98	C=O	NH of Arg105	2.56
		Phenyl ring of indole	Phenyl ring of Tyr67	Arene–arene
10a	−13.62	CN	CO of Tyr67	2.96
		NH of the triazine ring	C=N of Arg105	3.11
		Phenyl ring of indole	Phenyl ring of Tyr67	Arene–arene
10b	−16.01	CO of the triazine ring	CO of Try67	2.75
		NH of the triazine ring		3.02
10c	−11.99	NH of the triazine ring	CO of Glu95	2.99
		CN	C=N of Arg88	3.46
		S=O	NH of Arg88	2.57
		Phenyl ring of indole	NH of Arg88	Arene–H bond

Figure 6



(a) Docked conformation alignment of **5c** and its original cocrystallized ligand in the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L)-binding site. (b) Simplified structure showing an interaction between **5c** and the amino acid residues in the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L) active site.

Figure 7



(a) Docked conformation alignment of **9c** and its original cocrystallized ligand in the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L)-binding site. (b) Simplified structure showing an interaction between **9c** and the amino acid residues in the CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L) active site.

We can conclude that biological results were supported by docking results, which suggested that compounds **5c** and **9c** significantly inhibited the expression levels of the *BCL*-2 gene by a likeness approach that has 2W3L inhibitory activity.

Conclusion

A new series of triazolopyridines **3a-c,** diaminopyridines **4a-c**, pyridine diacetamides **5a-c**, triazolo[1,5-*a*]pyridines **6a-c-8a-c**, pyrido[1,2-*b*][1,2,4] triazines **9a-c, 10a-c**, pyrazoles **11a-c**, **12a-c**, and pyrimidine **13a-c-15a-c** derivatives incorporated into *N*-substituted sulfonyl indoles at their 3-positions were prepared. The newly synthesized compounds were tested for their *in-vitro* cytotoxic activity against the HepG2 cell line. Compounds **3c**, **4b**, **4c**, **5c**, **6c**, **9c**, **10a**, **10b**, and **10c**, which showed promising IC_{50} values, were chosen for the study of their pro-apoptotic effects. Compounds **4c**, **5c**, **9c**, **10a**, **10b**, and **10c** significantly inhibited the expression levels of the *BCL*-2 gene. The binding mode of the most promising proapoptotic compounds was carried out by docking with CHIMAERIC *BCL2*-XL protein (PDB ID: 2W3L). The results indicated that compounds **5c** and **9c** showed a good fit within the pocket site of the protein molecular surface and had a minimum binding energy of −20.29 and −18.98 kJ/mol, respectively, in comparison with the cocrystallized ligand, which is in agreement with the experimental result of a pro-apoptotic effect.

Therefore, they might be considered good inhibitors of CHIMAERIC *BCL2*-XL protein and consequently have a high proapoptotic activity.

Acknowledgements

The authors are grateful to the Micro-analytical Unit, National Research Centre, Giza, Egypt, for carrying out elemental analyses and determination of spectral data.

Conflicts of interest

None declared.

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