Design, synthesis, and biological evaluation of novel ciprofloxacin derivatives as potential anticancer agents targeting topoisomerase II enzyme

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ABSTRACT

A series of novel ciprofloxacin (CP) derivatives substituted at the N-4 position with biologically active moieties were designed and synthesised. 14 compounds were 1.02- to 8.66-fold more potent than doxorubicin against T-24 cancer cells. Ten compounds were 1.2- to 7.1-fold more potent than doxorubicin against PC-3 cancer cells. The most potent compounds 6, 7a, 7b, 8a, 9a, and 10c showed significant Topo II inhibitory activity (83–90% at 100 μM concentration). Compounds 6, 8a, and 10c were 1.01- to 2.32-fold more potent than doxorubicin. Compounds 6 and 8a induced apoptosis in T-24 (16.8- and 20.1-fold, respectively compared to control). This evidence was supported by an increase in the level of apoptotic caspase-3 (5.23- and 7.6-fold, sequentially). Both compounds arrested the cell cycle in the S phase in T-24 cancer cells while in PC-3 cancer cells the two compounds arrested the cell cycle in the G1 phase. Molecular docking simulations of compounds 6 and 8a into the Topo II active site rationalised their remarkable Topo II inhibitory activity.

GRAPHICAL ABSTRACT

Introduction

Cancer is a very dangerous and life-threatening disease, it is considered one of the most prevalent diseases in the world. The defining characteristic of cancer is metastasis, the leading cause of death from cancer. Many antitumor agents are commercially available, but the emergence of acquired drug resistance with severe side effects of these clinically used anticancer drugs poses serious barriers to effective chemotherapy. Therefore, it is recommended to rationally develop new anticancer drugs with fewer side effects.

DNA topological problems arise from the intertwined nature of the DNA double helix structure, which causes tangles and supercoiling of the DNA duplex during the DNA replication and transcription. DNA supercoiling results in torsion that impair the function of DNA or RNA polymerases. Type II topoisomerase enzyme (Topo II) prevents and corrects these types of topological problems via transient double-stranded breaks, causing DNA metabolism to proceed, allowing the cell to efficiently replicate so enabling cellular division and vitality. The role of divalent Mg ions in Topo II-mediated reactions was recognised as an implication in enzyme-mediated...
DNA cleavage reactions. (2) participation in ATPase reactions and functions by providing the enzyme with magnesium–ATP substrate. Topo II enzyme inhibition leads to apoptosis and cell death, therefore, it is considered a valid strategy in cancer therapy. The presence of topoisomerase enzyme in both mammalian and bacterial cells makes it a pronounced target for antibacterial and anticancer drugs. Recently, mammalian Topo II is considered a critical target for anticancer drug development. Among fluoroquinolones, is distinguished by strong inhibition of both prokaryotic and eukaryotic cells, due to the similarities between the prokaryotic and eukaryotic topoisomerases. A similar mechanism of action characterises several clinically important antitumor agents such as etoposide, doxorubicin, amarscine, or mitoxantrone. Recently, a great deal of work has been devoted to the antiproliferative activity of fluoroquinolones and several studies proved them as potent cytotoxic agents. Ciprofloxacin (CP), a broad-spectrum fluoroquinolone antibiotic, showed anti-proliferative activity against strains of human cancer cells. CP has been reported to pile up in urine and prostate tissues, therefore it is a privileged candidate for the treatment of bladder, and prostate cancers. CP, among fluoroquinolones, is distinguished by strong inhibition of Topo II. Additionally, it can induce the intrinsic apoptotic pathway by creating a double-stranded break in DNA or cell cycle arrest in the S/G2 phase. Thus, CP serves as a unique scaffold for the development of novel anticancer agents. The SAR studies uncovered that fluorene amine, the 1-alkyl, and 1, 4-dihydro-4-oxo-quinoline-3-carboxylic acid are the fundamental pharmacophore lineaments for CP anticancer activity.

Several studies have been conducted to determine the cytotoxic structural features of CP on eukaryotic cells. These studies changed the activity of fluoroquinolones from antibacterial to antitumor activity. Topo II inhibitory activity and pharmacokinetic features of CP are greatly affected by the modification at the C-7 position of CP which diminished the zwitterion character and greatly influenced the hydrophilicity nature leading to toxic structural features of CP on eukaryotic cells. These studies changed the activity of fluoroquinolones from antibacterial to antitumor activity. Inspired by all these findings, we have designed a series of novel CP derivatives with essential pharmacophoric features for CP anticancer activity.

**Materials and methods**

**Chemistry**

**General**

Melting points were determined on a Griffin apparatus and were uncorrected. Shimadzu IR 435 spectrophotometer (Shimadzu Corp., Kyoto, Japan), Faculty of Pharmacy, Cairo University, Cairo, Egypt, was used to record IR spectra, values were represented in cm⁻¹. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectra were recorded in ppm on the δ scale and coupling constants (J) were given in Hz on Bruker 400 MHz (Bruker Corp., Billerica, MA, USA) spectrophotometer, Faculty of Pharmacy, Cairo University, Cairo, Egypt. Tetra-methylysilane (TMS) was used as an internal standard. Progress of the reactions was monitored by TLC using pre-coated aluminium sheet silica gel MERCK 60F 254 and was visualised by a UV lamp.

**Procedure for the preparation of 1-cyclopropyl-7-(4-(2-ethoxy-2-oxoethyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1).** A mixture of ciprofloxacin (1.56 g, 0.005 mol), ethyl chloroacetate (0.61 g, 0.005 mol) and trimethylamine (10 g, 0.1 mol) in dimethylformamide (50 ml) was heated under reflux for 6 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and crystallised from ethanol to give compound 1. MP 190–192 °C; yield 86%.

**Procedure for the preparation of 1-cyclopropyl-6-fluoro-7-(4-(2-hydrazinyl-2-oxoethyl) piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (2).** A mixture of ester derivative (2.08 g, 0.005 mol) and hydrazine hydrate 99% (1.26 g, 0.025 mol) in absolute ethanol (5 ml) was heated under reflux for 6 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and crystallised from ethanol to give compound 2. MP 226–228 °C (as reported); yield: 75%.

**Procedure for the preparation of 1-cyclopropyl-6-fluoro-7-(4-(2-(3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (3).** A mixture of the hydrazinyl derivative (0.50 g, 0.001 mol) and ethyl acetooacetate (0.13 g, 0.001 mol) in absolute ethanol (5 ml) was heated under reflux for 6 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and crystallised from ethanol to give compound 3. MP 188–190 °C; yield: 60%; IR (KBr νmax: 3549 (OH), 3093 (C–H arom.), 2978 (C–H aliph.), 1730, 1720 (C = O) cm⁻¹. ¹H NMR (DMSO-d₆): δ 1.18–1.20 (m, 2H, CH₂ cyclopropyl), 1.30–1.35 (m, 2H,
CH₂ cyclopropyl), 2.47 (s, 3H, CH₃C=N), 2.70 – 2.75 (m, 4H, 2CH₂ piperazine), 3.30 – 3.40 (m, 4H, 2CH₂ piperazine), 3.40 – 3.50 (m, 1H, CH cyclopropyl), 3.80 (s, 2H, CH₂ pyrazolone), 4.30 (s, 2H, N-CH₂CO), 7.54 (d, 1H, J = 8 Hz, ArH), 7.85 (d, 1H, J = 13.6 Hz, ArH), 8.60 (s, 1H, C₂-H), 15.17 (s, 1H, COOH, D₂O exchangeable) ppm; ¹³CN M R (DMSO-d₆): δ 8.0, 14.6, 36.3, 49.8, 52.0, 58.6, 60.3, 107.1, 111.4, 119.0, 139.6, 145.5, 148.4, 152.2, 154.7, 166.3, 170.3, 176.7–176.8 ppm. Anal. Calcd. for C₂₃H₂₄FN₅O₅ (469.47): C, 58.84; H, 5.15; N, 14.92. Found: C, 58.93; H, 5.19; N, 15.22.

**Procedure for the preparation of 1-cyclopropyl-6-fluoro-7-(4-(2-(5-imino-3-oxopyrazolidin-1-yl)-2-oxoethyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (4).** A mixture of hydrazinyl derivative 2 (0.50 g, 0.001 mol) and ethyl cyanoacetate (0.11 g, 0.001 mol) in glacial acetic acid (10 ml) was heated under reflux for 5 h. The reaction mixture was filtered while hot, the filtrate was left to cool, and the separated solid was filtered, washed with water (15 ml) dried and recrystallised from ethanol to give compound 4. MP 181–183°C; yield 37%; IR (KBr) νmax 3541 (OH), 3437, 3417 (2NH), 3059 (C-H aliph.), 2870 (C-H aliph.), 1728, 1720 (C=O) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.25–1.40 (m, 2H, CH₂ cyclopropyl), 1.60–1.65 (m, 2H, CH₂ cyclopropyl), 2.80–2.95 (m, 4H, 2CH₂ piperazine), 3.36–3.41 (m, 4H, 2CH₂ piperazine), 3.44 (s, 2H, NCH₂O), 3.80–3.90 (m, 1H, CH cyclopropyl), 3.86 (s, 2H, CH₂ pyrazolone) 7.57 (d, 1H, ArH), 7.90 (d, 1H, J = 8 Hz, ArH), 8.66 (s, 1H, C₂-H), 9.68 (s, 1H, NH, D₂O exchangeable), 9.75 (s, 1H, NH, D₂O exchangeable), 15.19 (s, 1H, COOH D₂O exchangeable) ppm; ¹³CN M R (DMSO-d₆): δ 5.9, 12.5, 24.0, 34.2, 43.7, 47.5, 104.6, 109.3, 115.3, 116.8, 122.5, 137.5, 143.4, 146.2, 163.6, 164.2, 168.2, 169.5, 174.6 ppm. Anal. Calcd. for C₂₂H₂₃FN₆O₅ (470.45): C, 56.17; H, 4.93; N, 17.86. Found: C, 56.40; H, 4.81; N, 18.05.

**Procedure for the preparation of 1-cyclopropyl-7-(4-(2-(3,5-dimethyl-1H-pyrazol-1-yl)-2-oxoethyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5).** A mixture hydrazinyl derivative 2 (0.50 g, 0.001 mol) and acetylacetone (0.1 g, 0.001 mol) in absolute ethanol (5 ml) was heated under reflux for 6 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and crystallised from ethanol to give compound 5. MP: 185–187°C; yield: 60%; IR (KBr) νmax 3444 (OH), 3055 (C-H arom.), 2912 (C-H aliph.), 1730, 1728, 1720 (C=O) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.15–1.20 (m, 2H, CH₂ cyclopropyl), 1.30–1.35 (m, 2H, CH₂ cyclopropyl), 1.60–1.65 (m, 2H, CH₂ cyclopropyl), 2.80–2.95 (m, 4H, 2CH₂ piperazine), 3.36–3.41 (m, 4H, 2CH₂ piperazine), 3.44 (s, 2H, NCH₂O), 3.80–3.90 (m, 1H, CH cyclopropyl), 3.86 (s, 2H, CH₂ pyrazolone) 7.57 (d, 1H, ArH), 7.90 (d, 1H, J = 8 Hz, ArH), 8.66 (s, 1H, C₂-H), 9.68 (s, 1H, NH, D₂O exchangeable), 9.75 (s, 1H, NH, D₂O exchangeable), 15.19 (s, 1H, COOH D₂O exchangeable) ppm; ¹³CN M R (DMSO-d₆): δ 5.9, 12.5, 24.0, 34.2, 43.7, 47.5, 104.6, 109.3, 115.3, 116.8, 122.5, 137.5, 143.4, 146.2, 163.6, 164.2, 168.2, 169.5, 174.6 ppm. Anal. Calcd. for C₂₂H₂₃FN₆O₅ (470.45): C, 56.17; H, 4.93; N, 17.86. Found: C, 56.40; H, 4.81; N, 18.05.

**Figure 1.** Structures of CP and potent anticancer CP derivatives I–III.

**Figure 2.** Potent Topo II inhibitors.
cyclopropyl), 2.75–2.80 (m, 7H, 2CH2, CH3), 2.90 (s, 3H, CH3), 3.30–3.35 (m, 4H, 2CH2 piperazine), 3.40–3.50 (m, 1H, CH cyclopentyl), 3.80 (s, 2H, CH2CO), 7.55 (d, 1H, ArH), 7.88 (d, 1H, ArH), 7.90 (s, 1H, CH pyrazole), 8.63 (s, 1H, C2-H), 15.16 (s, 1H, COOH, D2O exchangeable) ppm; 13C NMR (DMSO-d6): δ 8.0, 14.6, 19.0, 36.3, 49.8, 52.0, 58.6, 106.8, 107.1, 111.2, 111.4, 118.9, 139.6, 145.6, 148.3, 152.2, 154.6, 166.4, 168.5, 170.3, 176.7 ppm. Calcd. for C24H26FN5O4 (467.49): C, 61.66; H, 5.61; N, 14.68. Found: C, 61.92; H, 5.72; N, 14.17.

Procedure for the preparation of 1-cyclopropyl-7-(4-(2-(2-(1-(4-Aminophenyl)ethylidene)hydrazinyl)-2-oxoethyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7a). A mixture of hydrazinyl derivative 2 (0.20g, 0.005 mol) in sodium ethoxide (0.034 g atomic weight of sodium in 17 ml absolute ethanol), diethyl malonate (0.1 g, 0.001 mol) and glacial acetic acid (1 ml) in absolute ethanol (5 ml) was heated under reflux for 6 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and crystallised from ethanol to give compound 7a. M.P.: 256–258 ºC; yield: 65%; IR (KBr) vmax 3497 (OH), 3417 (NH), 3043 (C-H arorn.), 2981, 2962 (C-H aliph.) cm⁻¹; 1H NMR (DMSO-d6): δ 1.18–1.25 (m, 2H, CH2 cyclopentyl), 1.31–1.33 (m, 2H, CH2 cyclopentyl), 2.91–2.93 (m, 2H, CH2 cyclohexylidene) 3.24–3.25 (m, 4H, CH2 cyclohexylidene), 3.40–3.50 (m, 1H, CH cyclopentyl), 3.60–3.70 (m, 4H, CH2 cyclohexylidene), 3.82 (s, 2H, CH2CO) 7.55 (d, 1H, J = 8 Hz, ArH), 7.87 (d, 1H, J = 12 Hz, ArH), 8.13 (s, 1H, CH, D2O exchangeable), 8.65 (s, 1H, C2-H) ppm; 13C NMR (DMSO-d6): δ 8.0, 19.0, 34.2, 36.2, 45.8, 51.0, 51.1, 57.0, 77.0, 106.4, 107.2, 111.3, 118.7, 139.6, 146.1, 148.2, 166.4, 152.1, 154.6, 176.7 ppm. Anal. Calcd. for C22H22FN5O6 (471.44): C, 61.66; H, 5.61; N, 14.17. Found: C, 61.92; H, 5.72; N, 14.16.

General procedure for the preparation of 7-(4-(1-(4-Chlorophenyl)ethylidene)hydrazinyl)-2-oxoethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7b). M.P.: 178–180 ºC; yield 75%; IR (KBr) vmax 3444 (OH), 3298 (NH), 3078 (C-H arorn.), 2924, 2827 (C-H aliph.) cm⁻¹; 1H NMR (DMSO-d6): δ 1.19–1.23 (m, 2H, CH2 cyclopentyl), 1.33–1.40 (m, 2H, CH2 cyclopentyl) 2.18 (s, 3H, CH3), 2.25–2.30 (m, 4H, CH2 cyclopentyl), 2.70–2.90 (m, 4H, CH2 cyclopentyl), 3.05 (s, 2H, CH2CO), 3.78–3.80 (m, 1H, CH cyclopentyl), 7.50–7.52 (m, 3H, ArH), 7.82–8.00 (m, 3H, ArH), 8.6 (s, 1H, C2-H), 10.42, 10.66 (2s, 1H, NH, D2O exchangeable), 15.22 (s, 1H, COOH, D2O exchangeable) ppm. Anal. Calcd. for C22H22ClFN5O6 (539.99): C, 60.06; H, 5.04; N, 12.97. Found: C, 60.13; H, 5.18; N, 12.92.

Figure 3. Design strategy for the syntheised CP hybrids.
NH₂, D₂O exchangeable), 7.11 (s, 1H, NH, D₂O exchangeable) 7.54 (d, 3H, ArH), 7.94 (d, 3H, ArH), 8.68 (s, 1H, C₂-H), 11.23 (s, 1H, OH exchangeable by D₂O) ppm. Anal. Calcd. for C₂₇H₂₆FN₆O₅S (532.56): C, 56.20; H, 5.52; N, 13.64. Found: C, 56.20; H, 5.52; N, 13.64.

1-Cyclopropyl-6-fluoro-7-(4-(2-oxo-2-(1-(2-hydroxyphenyl)ethylidene)-hydrazinyl)-2-oxoethyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7d). M.P.: 166–168; yield 80%; IR (KBr) νmax: 3441 (OH), 3187 (C–H arom.), 2974 (C–H aliph.), 1730, 1720, 1705 (C=O) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.10–1.20 (m, 2H, CH₂ cyclopropyl), 1.25–1.30 (m, 2H, CH₂ cyclopropyl), 2.40 (s, 3H, CH₃), 2.60–2.65 (m, 4H, 2CH₂ piperazine), 2.70–2.80 (m, 4H, 2CH₂ piperazine), 3.27 (s, 2H, CH₂ CO), 3.35–3.40 (m, 1H, CH cyclopropyl), 7.05–7.15 (m, 2H, ArH), 7.50–7.70 (m, 2H, ArH), 7.95 (d, 1H, ArH), 8.65 (s, 1H, C₂-H), 8.99, 9.67 (2s, 1H, NH, D₂O exchangeable), 15.19 (s, 1H, COOH, D₂O exchangeable) ppm. ¹³C NMR (DMSO-d₆): δ 8.0, 14.0, 14.6, 14.7, 49.9, 52.6, 106.8, 107.7, 111.2, 111.8, 112.9, 128.5, 134.5, 143.5, 144.0, 145.6, 148.3, 154.6, 168.5, 171.5, 176.7 ppm. Anal. Calcd. for C₂₇H₂₆FN₆O₅S (532.56): C, 58.70; H, 5.12; N, 13.64. Found: C, 58.63; H, 5.15; N, 13.72.

General procedure for the preparation of 7-(4-[(2-oxo-2-[(5-Chlorothiophen-2-yl)ethylidene]hydrazinyl)-2-oxoethyl]piperazin-1-yl)-1-cyclopropyl-6-fluoro-7-(4-(2-oxo-2-(1-(2-hydroxyphenyl)ethylidene)hydrazinyl)ethyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (8a-c). A mixture of hydrazinyl derivative 2 (0.50 g, 0.001 mol), substituted phenyl isocyanate or phenyl isothiocyanate (0.001 mol), and glacial acetic acid (1 ml) in absolute ethanol (15 ml) was heated under reflux for 7 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and recrystallised from ethanol to give compounds 8a–b.

7-(4-(2-[(4-Chloro-3-(trifluoromethyl)phenyl)carbamoyl]hydrazinyl)-2-oxoethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (9a-c). A mixture of hydrazinyl derivative 2 (0.50 g, 0.001 mol), substituted phenyl isocyanate or phenyl isothiocyanate (0.001 mol), and glacial acetic acid (1 ml) in absolute ethanol (15 ml) was heated under reflux for 7 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and recrystallised from ethanol to give compounds 9a–c.
7-(4-(2-(2-Chloro-6-methylphenyl)carbamoyl)hydrazinyl)-2-oxoethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (9b). M.P.: 285–287; yield 83%; IR (KBr) \( \nu_{\text{max}} \): 3572 (OH), 3441 (NH), 3051 (C–H aliph.), 1730, 1728 (C = O) cm\(^{-1}\). -H NMR (DMSO-d\(_6\)): \( \delta \) 1.15–1.25 (m, 4H, 2CH\(_2\) cyclopropyl), 2.20–2.25 (m, 11H, 4CH\(_2\) piperazine, CH\(_3\)), 3.34 (s, 2H, 7H-CO), 4.80–4.90 (m, 1H, CH cyclopropyl), 7.17–7.24 (m, 3H, ArH), 7.33–7.35 (m, 3H, ArH), 8.94 (brs, 3H, 3NH, D\(_2\)O exchangeable). \( \nu_{\text{C–O}} \) cm\(^{-1}\): 1762.5, 1762.1, 1762.6, 1762.7 ppm. Anal. Calcd. for C\(_{37}\)H\(_{34}\)F\(_6\)N\(_8\)O\(_7\): C, 57.24; H, 4.80; N, 14.02.

1-Cyclopropyl-6-fluoro-7-(4-(2-(1,3-dioxoisoindolin-2-ylamino)-2-oxoethyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (10a–c)

A mixture of hydrazine derivative 2 (0.50 g, 0.001 mol), anhydrous potassium carbonate (0.41 g, 0.003 mol) and substituted phenacyl bromide (0.001 mol) in dry benzene (8 ml) was heated under reflux for 24 h. The reaction mixture was filtered while hot. The residue was washed twice with water (20 ml), dried, and recrystallised from ethanol to give compound 10a–c.

1-Cyclopropyl-6-fluoro-7-(4-(2-(2-(2-oxoethyl)hydrazinyl)ethyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (10a). M.P.: 183–185°C; yield: 45%; (KBr) \( \nu_{\text{max}} \): 3500 (OH), 3402 (NH), 3008 (C–H aliph.), 2916 (C–H aliph.), 1732, 1700, 1697 (C = O) cm\(^{-1}\). -H NMR (DMSO-d\(_6\)): \( \delta \) 1.11–1.18 (m, 2H, CH\(_2\) cyclopropyl), 1.30–1.32 (m, 2H, CH\(_2\) cyclopropyl), 2.75–2.79 (m, 2H, CH\(_2\) piperazine), 2.90–2.95 (m, 2H, CH\(_2\) piperazine), 3.40 (s, 2H, CH\(_2\)CO), 3.45–3.50 (m, 2H, CH\(_2\) piperazine), 3.60–3.70 (m, 2H, CH\(_2\) piperazine), 3.80–3.90 (m, 1H, CH cyclopropyl), 4.00 (s, 2H, CH\(_2\)CO), 5.61 (s, 1H, NH, D\(_2\)O exchangeable), 7.37–8.05 (m, 7H, ArH), 8.56 (s, 1H, C\(_2\)H–H), 8.66 (s, 1H, NH, D\(_2\)O exchangeable). ppm. \( \nu_{\text{C–O}} \) cm\(^{-1}\): 13C NMR (DMSO-d\(_6\)): \( \delta \) 7.9, 17.0, 26.0, 36.5, 46.2, 50.0, 102.0, 111.0, 116.5, 126.0, 127.4, 128.5, 129.4, 143.0, 144.5, 150.5, 161.0, 166.6, 174.5 ppm. Anal. Calcd. for C\(_{27}\)H\(_{25}\)F\(_2\)N\(_8\)O\(_6\): 716.55: C, 57.24; H, 4.80; N, 14.02.

Procedure for the preparation of 1-cyclopropyl-7-(4-(2-(2-(2-oxoethyl)hydrazinyl)ethyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (11). A mixture of hydrazinyl derivative 2 (0.50 g, 0.001 mol), succinic anhydride (0.10 g, 0.001 mol), and anhydrous sodium acetate (0.117 g, 0.0015 ml) in glacial acetic acid (10 ml) was heated under reflux for 5 h. The reaction mixture was concentrated to half its volume and allowed to cool, the separated solid was filtered, washed with cold ethanol, dried, and recrystallised from acetic acid to give compound 11. M.P.: 184–186°C; yield: 55%; (KBr) \( \nu_{\text{max}} \): 3441 (OH), 3224 (NH), 3016 (C–H aliph.), 2947 (C–H aliph.), 1720, 1712, 1700 (C = O) cm\(^{-1}\). -H NMR (DMSO-d\(_6\)): \( \delta \) 1.11–1.18 (m, 2H, CH\(_2\) cyclopropyl), 1.31–1.33 (m, 2H, CH\(_2\) cyclopropyl), 2.13–2.24 (s, 4H, CH\(_2\) pyrroline), 2.70–2.80 (m, 4H, CH\(_2\) piperazine), 3.10 (s, 2H, CH\(_2\)CO), 3.30–3.40 (m, 4H, CH\(_2\) cyclopropyl), 8.66 (s, 1H, C\(_2\)H–H), and 9.68 (s, 1H, NH, D\(_2\)O exchangeable). ppm. \( \nu_{\text{C–O}} \) cm\(^{-1}\): 13C NMR (DMSO-d\(_6\)): \( \delta \) 8.0, 19.5, 20.8, 26.6, 28.7, 29.8, 106.6, 107.3, 111.2, 111.4, 119.0, 139.5, 145.5, 148.2, 154.6, 154.6, 154.6, 154.6, 166.6, 166.6, 166.6, 176.4 ppm. Anal. Calcd. for C\(_{27}\)H\(_{25}\)F\(_2\)N\(_8\)O\(_6\): 756.55: C, 57.24; H, 4.80; N, 14.02.

Procedure for the preparation of 1-cyclopropyl-7-(4-(2-(2-(2-oxoethyl)hydrazinyl)ethyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12). A mixture of compound 2 (0.50 g, 0.001 mol), phthalic anhydride (0.15 g, 0.001 mol), and anhydrous sodium acetate (0.117 g, 0.0015 ml) in glacial acetic acid (10 ml) was heated under reflux for 5 h. The reaction mixture was concentrated to half its volume and allowed to cool, the separated solid was filtered, washed with cold ethanol, dried, and recrystallised from ethanol to give compound 12.
recrystallised from acetic acid to give compound 12. M.P: 190–192 °C; yield: 50%; IR (KBr) ν max: 3441 (OH), 3224 (NH), 3012 (C–H aliph.), 2897 (C–H aliph.), 1720, 1700 (C = O) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.10–1.15 (m, 2H, CH₂ cyclopropyl), 1.20–1.30 (m, 2H, CH₂ cyclopropyl), 2.65–2.75 (m, 4H, 2CH₂ piperazine), 3.11 (s, 2H, CH₂CO), 3.25–3.35 (m, 4H, 2CH₂ piperazine), 3.75–3.80 (m, 1H, CH cyclopropyl), 7.36–8.15 (m, 6H, ArH), 8.63 (s, 1H, C2-H), and 9.74 (s, 1H, NH, D₂O exchangeable) ppm. Analytical data for C₂₂H₂₃FN₆O₅S (502.52): C, 52.58; H, 4.61; N, 16.72. Found: C, 52.79; H, 4.58; N, 13.11.

Procedure for the preparation of 7-(4-(2-(carbamothioylhydrazinyl)-2-oxoethyl) piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (13). A mixture of hydrazinyl derivative 2 (0.050 g, 0.001 mol), potassium thiocyanate (0.194 g, 0.002 mol), and concentrated hydrochloric acid (1 ml) in absolute ethanol (15 ml) was heated under reflux for 6 h. The reaction mixture was cooled, and the separated solid was filtered, dried, and recrystallised from ethanol to give compound 13. M.P: 188–200 °C; yield: 65%; IR (KBr) ν max: 3417 (OH), 3271, 3236 (NH, NH₂), 3001 (C–H arom.), 2947 (C–H aliph.), 1747, 1728 (C = O) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.19–1.21 (m, 2H, CH₂ cyclopropyl), 1.34–1.35 (m, 2H, CH₂ cyclopropyl), 3.30–3.50 (m, 8H, 4CH₂ piperazine), 3.76 (s, 2H, CH₂CO), 3.87–3.89 (m, 1H, CH cyclopropyl), 7.20 (s, 1H, NH, D₂O exchangeable), 7.30 (s, 2H, NH₂, D₂O exchangeable), 7.40 (s, 1H, NH, D₂O exchangeable), 7.63 (d, 1H, J = 8 Hz, ArH), 7.96 (d, 1H, J = 13 Hz, ArH), 8.68 (s, 1H, C2-H), and 15.20 (s, 1H, COOH, D₂O exchangeable) ppm; ¹³C NMR (DMSO-d₆): δ 8.0, 14.5, 17.0, 51.8, 56.1, 107.2, 130.0, 148.4, 148.5, 152.0, 152.1, 154.5, 166.2, 166.3, 168.5, 176.7 ppm. Analytical data for C₁₄H₁₃FN₄O₇S (562.18): C, 59.78; H, 4.84; N, 14.94.

Biological activity

Cell culture protocol

Human bladder cancer (T-24) and human prostate carcinoma (PC-3) cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA USA). Cells were maintained in Dulbecco’s modified Eagle medium with 10% foetal bovine serum at 37 °C and 5% CO₂. All the operations were carried out under strict aseptic conditions. The culture medium was removed to a centrifuge tube containing 9 ml complete culture medium and spanned at approximately 125 × g for 5 to 7 min. The cell layer was rinsed with 0.25% (w/v) trypsin-0.53 μM EDTA solution by which all traces of serum-containing inhibitor were removed. Trypsin EDTA solution 2.0 to 3.0 ml was added to a flask and cells were monitored under an inverted microscope until the cell layer was dispersed (usually within 5–15 min). A complete growth medium was added (6.0–8.0 ml), and cells were aspirated by gentle pipetting. The cell pellet was suspended with the recommended complete medium and dispensed into a 75 cm² culture flask. The culture vessel containing the complete growth medium was placed in the incubator for 24 h to 37 °C. The cells were treated with different concentrations (0.39, 1.60, 6.25, 25, and 100 μg/ml) of each of the test compounds or doxorubicin, followed by incubation for 48 h at 37 °C, then the plates were examined under the inverted microscope and finally the MTT assay was carried out.

Cell viability assay

Anti-cancer activity of the newly synthesised compounds was evaluated in vitro against both T-24 and PC-3 cell lines according to the MTT method. Cells were seeded into 96-well plates (flat bottom) at a density of 10 000 cells/well for 24 h. The vial of MTT to be used was reconstituted with 3 ml of medium or balanced salt solution without phenol red and serum. then, a reconstituted MTT vial was added in an amount equal to 10% of the culture medium volume. Cultures were incubated for 2–4 h depending on the cell type and maximum cell density and removed from the incubator and the formazan crystals were dissolved via the addition of an amount of MTT solubilisation solution (M-8910) equal to the original culture medium volume. ROBONIK P2000 spectrophotometer at a wavelength of 570 nm was used to measure the colour intensity spectrophotometrically. The survival curve of both T-24 and PC-3 cells was obtained by plotting the percentage of surviving cells against the drug after each compound. The IC₅₀ value for each test compound and the reference drug doxorubicin was calculated.
In vitro DNA topo II-mediated relaxation assay

DNA Topo II inhibitory activities of the targeted compounds were measured as follows. A mixture of 100 ng of supercoiled pBR322 plasmid DNA (Fermentas, USA) and 2 units of human DNA Topo II (USB Corp., USA) was incubated with and without the prepared compounds in the assay buffer (10 μM Tris-HCl pH 7.9) containing 50 μM NaCl, 4 μM MgCl₂, 1 μM EDTA, 1 μM ATP, and 15 mg/ml bovine serum albumin for 30 min at 37°C. The reaction in a final volume of 10 μl was terminated by the addition of 4 μl of 6 μM EDTA. DNA samples were then electrophoresed on 1% agarose gel containing 0.5 μg/ml ethidium bromide in gel and buffer at high voltage (100-250 v) until the dye front has migrated about 4-6 cm down the gel, with a running buffer of TAE Tris-acetate–EDTA (TAE). Gels were stained for 15 min in water. DNA bands were visualised by transillumination with UV light and quantitated using AlphaFsoft software (Alpha Innotech Corporation).

Cell cycle analysis of compounds 6 and 8a

The cell cycle was done using a propidium iodide flow cytometry kit (Abcam, ab139418) according to the manufacturer’s instructions. T-24 and PC-3 cells were treated with IC₅₀ of compounds 6 and 8a for 24 h. After treatment, the cells were washed twice with ice-cold phosphate buffer saline, then centrifuged and fixed using ice-cold 66% ethanol. Ethanol was added with phosphate buffer saline re-suspended with 0.10 mg/ml, and stained with 200 μl propidium iodide. Cells were analysed by flow cytometry. The cell cycle distributions were calculated using cell–quest software (Becton Dickinson).

Apoptosis determination

Apoptosis was evaluated using Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) using the Annexin V-FITC/PI apoptosis detection kit (Biovision, Mountain View, CA # K101-25). According to the manufacturer’s instructions after staining the cells with annexin V fluorescein (FITC) isothiocyanate. Briefly, 1–5 × 10⁵ cells were exposed to compounds 6 and 8a at their IC₅₀ concentrations for 24 h. Cells were centrifuged and washed once with serum-containing media followed by resuspension in 500 μl of binding buffer. Then 5 μl Annexin V-FITC and 5 μl of (PI 50 mg/ml) were added and incubated for 5 min at room temperature in the dark. Analyses were performed using a flow cytometer (EX = 488 nm: Em = 530 nm) using a FITC signal detector and PI staining by a phycoerythrin emission signal detector.

Measurement of the effect of compounds 6 and 8a on the level of caspase-3 protein (a marker of apoptosis)

The level of the apoptotic marker caspase-3 was measured using The Invitrogen Caspase-3 (active) Human ELISA Kit. The procedure of the used kit was done according to the manufacturer’s instructions.

Molecular docking study

Molecular docking studies were performed using the Molecular Operating Environment (MOE, 2014.0901) software. All minimisations were performed using MOE with MMFF94x force field, and the partial and formal charges were calculated automatically. The X-ray crystallographic structures of topoisomerase IIα co-crystallised with DNA were downloaded from the protein data bank.

To prepare the enzyme for the docking study, first DNA chains and water molecules were removed, and then the protonate 3D protocol in MOE with default options was used. For the docking protocol, the triangle matcher placement method and London dG scoring function were used. Validation of the docking results was carried out by docking the reference topoisomerase inhibitor merbarone and comparing the results with a previously reported study. The MOE-validated setup was used to predict the binding interactions and affinity of the synthesised compounds at the active site.

Results and discussion

Chemistry

The synthetic approaches designed in this study for the synthesis of the target compounds are elucidated in Schemes 1–3. The primary starting compound 1, was prepared by reacting CP with ethyl chloroacetate in dimethylformamide in the presence of triethylamine. Compound 1 was reacted with hydrazine hydrate to afford compound 2.

Compound 3 was prepared by reacting hydrazinyl derivative 2 with ethyl cyanoacetate in absolute ethanol. The ¹H NMR spectrum of this compound displayed the disappearance of the signal corresponding to NH₂ of the parent compound 2 and the appearance of two singlet signals at δ 2.47 and 3.80 ppm corresponding to CH₃C–N and CH₂ pyrazolone protons, respectively.

CP derivative 4 was prepared by reacting compound 2 with ethyl cyanoacetate in glacial acetic acid. The ¹H NMR spectrum displayed a singlet signal at δ 3.86 ppm expressing the CH₂ pyrazolone protons. Compound 5 was obtained via refluxing CP hydrazinyl derivative 2 with acetylacetone in ethanol. The ¹H NMR spectrum displayed the disappearance of the signal corresponding to NH₂ of the parent compound 2 in addition to the presence of one singlet signal at δ 7.90 ppm corresponding to CH of pyrazole.

Compound 6 was obtained by reacting compound 2 with diethyl malonate in the presence of sodium ethoxide. The ¹H NMR spectrum of this compound revealed the appearance of a singlet signal at δ 2.90 ppm due to the CH₂ of pyrazolone.

In Scheme 2, the hydrazones 7a–f were obtained by reacting compound 2 with the appropriate ketone in ethanol in the presence of glacial acetic acid. The ¹H NMR and ¹³C NMR spectra of these compounds displayed the presence of signals corresponding to different alkyl or aryl groups which were not present in compound 2.

Refluxing compound 2 with the suitable isatin in absolute ethanol in the presence of glacial acetic acid afforded compounds 8a–b. The ¹H NMR and ¹³C NMR spectra of these compounds revealed the presence of different signals of indoline moieties.

Compounds 9a–c were prepared via reacting compound 2 with the appropriate phenyl isocyanates or phenyl isothiocyanate in absolute ethanol in the presence of glacial acetic acid. The ¹H NMR and ¹³C NMR spectra of these derivatives displayed the characteristic signals corresponding to different aryl moieties. Further structural evidence stemmed from the ¹H NMR spectra that showed the exchangeable singlet signals corresponding to NH protons.

CP derivatives 10a–c were prepared via reacting compound 2 with the suitable phenacyl bromide in dry benzene in the presence of potassium carbonate. The ¹H NMR and ¹³C NMR spectra of these derivatives showed the appearance of new signals corresponding to the added phenyl ring in addition to singlet signals that appeared at δ 3.30–3.40 ppm due to CH₂CO protons.

In Scheme 3, the reaction of compound 2 with succinic anhydride or phthalic anhydride in glacial acetic acid in the presence of anhydrous sodium acetate yielded compounds 11 and 12,
respectively. The $^1$H NMR and $^{13}$C NMR spectra of these compounds showed the appearance of new signals corresponding to pyrrolidine and isoindoline moieties.

Reacting compound 2 with potassium thiocyanate and concentrated hydrochloric acid in absolute ethanol afforded compound 13. $^{13}$C NMR spectrum revealed the appearance of (C=S) carbon at $\delta$ 176.7 ppm. Compound 14 was prepared via reacting derivative 13 with ethyl chloroacetate in ethanol. The $^1$H NMR spectrum of compound 14 revealed the disappearance of the exchangeable signal of NH$_2$ protons.

Finally, compound 15 was prepared by refluxing compound 13 with phenacyl bromide in absolute ethanol in the presence of anhydrous sodium acetate. The $^1$H NMR and $^{13}$C NMR spectra showed the expected signals corresponding to the phenyl group, which were not present in the starting compound 14. Additionally, the $^1$H NMR spectrum revealed the appearance of a single signal corresponding to CH of the thiazole ring at $\delta$ 8.31 ppm.

**Biological activity**

**Growth inhibition against human tumour cell lines**

In this study, all the newly synthesised CP derivatives were subjected to anticancer activity evaluation against bladder (T-24) and prostate cancer (PC-3) cell lines. The compounds were evaluated for their activity using 5 doses determinations (100 $\mu$g/ml, 25 $\mu$g/ml, 6.25 $\mu$g/ml, 1.60 $\mu$g/ml, and 0.39 $\mu$g/ml). Their half-maximal inhibitory concentration (IC$_{50}$) values were measured. Doxorubicin was chosen as a reference anticancer drug.

The test compounds showed anticancer activity against the T24 cell line with IC$_{50}$ values ranging from 3.36–366 $\mu$M. They
exhibited IC\textsubscript{50} values against the PC-3 cell line in the range of 3.25–159.24 \mu M (Table 1). Compounds 4–6, 7a–c, 7e, 8a, 9a–b, 10b–c, 14, and 15 showed 1.02- to 8.66-fold more potent anti-proliferative activity than the reference standard doxorubicin against T-24 cell line (Figure 4).

Compounds 7a–b, 7d, 8a, 9b–c, 10b–c, 14, and 15 exhibited 1.2- to 7.1-fold more potent anti-proliferative activity than doxorubicin against PC-3 cells line (Figure 5).

Regarding the activity towards bladder cancer, compounds 6, 7a, 7b, 8a, 9a, and 10c were the most potent among the synthesised CP derivatives.

It is worth mentioning that the T-24 bladder cancer cell line was more sensitive to the synthesised CP derivatives than the PC-3 prostate cancer cell line.

The Structure-activity correlation of the newly synthesised CP derivatives revealed that modification at the piperazinyl N-4 position of the CP scaffold resulted in variable potency. CP derivatives bearing pyrazole ring through acetyl spacer (4–6) showed potent anticancer activity. They showed more potency against bladder cancer cell line, compound 6 incorporating pyrazolidine-3,5-dione moiety was the most prominent among them. Regarding CP hydrazones 7a–e, compound 7a incorporating cyclohexylidene moiety showed marked potency against bladder and prostate cancer cell lines. Further analysis of these compounds revealed that hydrazones carrying substituted phenyl ring (7b–d) were more potent than their counterparts having thiophene ring (7e,f). An interesting phenomenon is that CP hydrazone 7d featuring ortho hydroxyl group on the phenyl ring showed the most potent antiproliferative activity among CP aryl hydrazones against prostate cancer. CP derivative 8a having the acylhydrazone scaffold with a benzene ring carrying a nearby NH proved to marked anti-proliferative activity against both cell lines. It was clear that the introduction of a bromine atom on the indoline scaffold (8b) had a bad impact on the anticancer activity. CP semicarbazide and thiosemicarbazide derivatives 9a–c possessed potent activity. It was noticed that compound 9a featuring the 4-chloro-3-trifluoromethylphenyl moiety group was the most potent. Reviewing compounds 10a–c, revealed that the substitution of phenyl moiety with electron withdrawing group in 10b and 10c significantly improved the anticancer activity against the two cell lines. The incorporation of monocyclic pyrrolidine or pyrrolidine fused with benzene in CP derivatives 11 and 12 resulted in lower anticancer activity. It is worth mentioning that, grafting thiazole moiety in derivatives 14 and 15 highly improved the antiproliferative activity.

Recombinant topoisomerase II inhibitory activities of compounds (6, 7a, 7b, 8a, 9a, and 10c)
The conversion of supercoiled plasmid DNA to relaxed DNA by recombinant Topo II was examined in the presence of each of the most potent compounds 6, 7a, 7b, 8a, 9a, and 10c for measuring their Topo II inhibitory activities. Well-known Topo II inhibitor doxorubicin was used as a positive control. The reaction products of Topo II relaxation assays were analysed by electrophoretic...
mobility and developed in ethidium bromide in the presence of UV light. The inhibitory activities were evaluated at 4 concentrations of 100, 10, 1, and 0.1 μM for all compounds as shown in (Figure 6). Topo II inhibitory activities of the tested compounds are summarised in (Table 2).

All test compounds showed significant Topo II inhibitory activity in the range of 83–90% at 100 μM concentration. While at 0.1 μM concentration, the range of Topo II inhibition was 13.7–32.5% (Table 2). The IC50 value of each compound was calculated. Compounds 6, 8a, and 10c were 1.01- to 2.32-fold more potent than doxorubicin (Table 3).

### Cell cycle analysis and detection of apoptosis

The most prominent compounds 6 and 8a that showed Topo II IC50 values in the sub-micro-molar range were further investigated in terms of their effects on apoptosis induction and cell cycle progression in both T-24 and PC-3 cell lines.

T-24 and PC-3 cells were treated with compounds 6 and 8a at their IC50 values for 24 h and their effects on the normal cell cycle profile and induction of apoptosis were recorded. Treatment of T-24 and PC-3 cells with compounds 6 and 8a resulted in an interference with the normal distribution of the cell cycle. In T-24 cell lines, both compounds 6 and 8a induced a significant increase in the percentage of cells at pre-G1 by 16.7- and 20.1-fold, respectively when compared to the control. Also, they showed an increase in the percentage of cells in the S phase by 1.4- and 1.11-fold, sequentially compared to the control (Figures 7–9). The accumulation of cells in the pre-G1 phase indicated that CP derivatives 6 and 8a induced tumour cell death and cytotoxicity via apoptosis. While the accumulation of the cells in the S phase might result from the cell cycle arrest of T-24 cells in the S phase.

In PC-3 cell lines, both compounds 6 and 8a induced an increase in the percentage of cells at the G1 phase by 1.16- and 1.27-fold, respectively when compared to the control (Figures 10–12). This was confirmed by a concomitant decrease in the percentage of cells in the G2/M phase, where both compounds 6 and 8a induced a decrease in the percentage of cells at the G2/M
Compounds 6 and 8a induced G1-phase cell cycle arrest of PC-3 cells at their IC50 concentrations. Topoisomerase inhibitors cause DNA damage which is related to G1/S and S phase arrest. Cell cycle arrest prevents the replication of damaged DNA. In PC-3 cell line compounds 6 and 8a probably arrested the cell cycle in the G1 phase by inhibition of Cyclin D–Cdks complex, however in the T-24 cell line, they might arrest the cell cycle in the S phase by the inhibition of both Cyclin D–Cdks complex and cyclins E1–E2/Cdk2, which promote G1/S transition.

Similar effects regarding cell cycle arrest at different phases by CP derivatives have been reported, which approved that CP is a promising framework for anticancer drug design and discovery. In 2018, two CP derivatives induced a significant increase in the percentage of cells at pre-G1 and G2/M phases by 13.9-, 8.8-fold, and 2.05-, 2.06-fold, respectively compared to the negative control in the UO-31 cell line.

Figure 4. Graphical representation for the half-maximal inhibitory concentration (IC50) of CP derivatives and doxorubicin after treatment for 24 h on bladder cancer cell line.

Figure 5. Graphical representation for half-maximal inhibitory concentration (IC50) of the target compounds and doxorubicin after treatment for 24 h on the prostate cancer cell line.

Figure 6. Recombinant Topo II inhibitory activities of compounds 6, 7a, 7b, 8a, 9a, and 10c at 100, 10, 1, 0.1 μM. Lane P: pBR322 DNA only; lane N: pBR322 DNA + Topo II; lanes (6, 7a, 7b, 8a, 9a and 10c): pBR322 DNA + Topo II + compounds 6, 7a, 7b, 8a, 9a and 10c.
In 2021, Abdel-Rahman et al. reported that a Mannich base CP derivative induced an increase in the percentage of cells at pre-G1 and G2/M phases by 17.9- and 4.1-fold, respectively, in the OVCAR-3 cell line compared to the negative control70.

**Apoptosis determination by annexin V-FITC assay**

To verify the ability of compounds 6 and 8a to induce apoptosis, a flow cytometry assay was performed using propidium iodide (PI), and immunofluorescent markers of the protein annexin-V. Dual staining for annexin-V and with PI provides the distinction between viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells71–73. Treatment of T-24 cells with compounds 6 and 8a at their IC50 concentrations resulted in a decrease in the percentage of viable cells. The results showed that compound 6 induced both early and late apoptosis with 16.27- and 83.38-fold more than the control, respectively, and induced total apoptosis and necrosis with 16.77- and 6.25-fold more than the control, respectively (Figure 13). While compound 8a induced both early and late apoptosis with 21.16- and 114.90-fold more than the control, respectively, and induced total apoptosis and necrosis with 20.1- and 4.28-fold more than the control, sequentially (Figure 14).

While treatment of PC-3 cells with compounds 6 and 8a at their IC50 concentrations resulted in a decrease in the percentage of viable cells. The results showed that compound 6 induced both

| Compounds | Percentage of inhibition* |
|-----------|--------------------------|
|           | 100 µM | 10 µM | 1 µM | 0.1 µM |
| 6         | 87.4   | 76.3  | 51.9 | 28.0   |
| 7a        | 83.6   | 55.9  | 27.9 | 13.7   |
| 7b        | 85.2   | 60.3  | 34.0 | 18.4   |
| 8a        | 91.6   | 77.4  | 50.3 | 32.5   |
| 9a        | 86.5   | 57.0  | 40.3 | 23.6   |
| 10c       | 90.2   | 72.6  | 37.7 | 24.8   |

*Percentage inhibition of Topo II activity = (Intensity of sample-treated DNA/Intensity of vehicle-treated control DNA) × 100.

Table 3. Topo II IC50 results of compounds (6, 7a, 7b, 8a, 9a, and 10c) compared to doxorubicin.

| Compound | IC50 (µM ±SD) |
|----------|---------------|
| 6        | 0.92 ± 0.05 |
| 7a       | 4.99 ± 0.27  |
| 7b       | 3.34 ± 0.18  |
| 8a       | 0.74 ± 0.04  |
| 9a       | 2.58 ± 0.14  |
| 10c      | 1.69 ± 0.09  |
| Doxorubicin | 1.72 ± 0.08 |

*The results given are the means of three experiments.

In 2021, Abdel-Rahman et al. reported that a Mannich base CP derivative induced an increase in the percentage of cells at pre-G1 and G2/M phases by 17.9- and 4.1-fold, respectively, in the OVCAR-3 cell line compared to the negative control70.

**Table 2.** Recombinant Topo II inhibitory activities of compounds (6, 7a, 7b, 8a, 9a, and 10c).

| Compounds | Percentage inhibition* |
|-----------|--------------------------|
|           | 100 µM | 10 µM | 1 µM | 0.1 µM |
| 6         | 87.4   | 76.3  | 51.9 | 28.0   |
| 7a        | 83.6   | 55.9  | 27.9 | 13.7   |
| 7b        | 85.2   | 60.3  | 34.0 | 18.4   |
| 8a        | 91.6   | 77.4  | 50.3 | 32.5   |
| 9a        | 86.5   | 57.0  | 40.3 | 23.6   |
| 10c       | 90.2   | 72.6  | 37.7 | 24.8   |

*Percentage inhibition of Topo II activity = (Intensity of sample-treated DNA/Intensity of vehicle-treated control DNA) × 100.
Figure 9. Bar presentation showing effects of compounds 6 (5.68 µM) and 8a (3.36 µM) on DNA-ploidy flow cytometric analysis of T-24 cells after 24 h.

Figure 10. Effect of compound 6 (92.16 µM) on DNA ploidy flow cytometric analysis of PC-3 cells after 24 h.

Figure 11. Effect of compound 8a (10.95 µM) on DNA-ploidy flow cytometric analysis of PC-3 cells after 24 h.
Figure 12. Bar presentation showing effects of compounds 6 (92.16 \mu M) and 8a (10.95 \mu M) on DNA-ploidy flow cytometric analysis of PC-3 cells after 24 h.

Figure 13. Representative dot plots of T-24 cells treated with 6 (5.68 \mu M) for 24 h and analysed by flow cytometry after double staining of the cells with annexin-V FITC and PI.

Figure 14. Representative dot plots of T-24 cells treated with 8a (3.36 \mu M) for 24 h and analysed by flow cytometry after double staining of the cells with annexin-V FITC and PI.
early and late apoptosis with 54.69- and 31.72-fold more than the control, respectively, and induced total apoptosis and necrosis with 13.02- and 1.62-fold more than the control, respectively (Figure 15). While compound 8a induced both early and late apoptosis with 41.15- and 76.77-fold more than the control, respectively, and induced total apoptosis and necrosis with 14.87- and 2.44-fold more than the control, sequentially (Figure 16).

Effect of compounds 6 and 8a on the level of active caspase-3 (key executioner of apoptosis)
Caspase-3 (a key effector enzyme) plays an important role in apoptosis since its activation leads to catalysing specific enzymes responsible for DNA fragmentation, which leads to cell death. Apoptosis induction in T-24 cells by compounds 6 and 8a was investigated via caspase 3, compared to doxorubicin as a reference drug.

Treatment of T-24 cells with compounds 6 and 8a at concentrations 5.68 and 3.36 \( \mu \)M, sequentially enhanced the level of active caspase-3 compared to the control (5.23- and 7.6-fold) (Table 4, Figure 17).

### Molecular docking study
Docking studies were carried out for compounds 6 and 8a which showed potent activity in the Topo II enzyme inhibition assay. We used topoisomerase II co-crystallised with DNA (PDB ID: 4FM9) for molecular docking of compounds 6, 8a, and merbarone. Docking of merbarone in the DNA binding site was carried out for...
validation of the molecular docking and it was compared with a previously reported study. Interestingly, the test compounds exerted favourable interactions with the Topo II enzyme active site, with almost the same binding pattern as merbarone. Merbarone showed coordinate bond interactions with Mg$^{2+}$ and H-bond interaction with amino acid Asp 543 (Figure 18).

**Figure 17.** Graphical representation for active caspase-3 assays of compounds 6 and 8a compared to doxorubicin as a positive control.

**Figure 18.** 3D interaction of merbarone with DNA binding site of topoisomerase IIα. Red dashed lines represent coordinate bond interactions with Mg$^{2+}$. Red tiny, dashed lines are hydrogen bonding interactions with amino acid Asp 543. Mg$^{2+}$ is shown as a nonbonded sphere (crimson red). Residues that are involved in hydrogen bonding are shown in the stick presentation.

**Figure 19.** 3D interaction of compound 6 with DNA binding site of topoisomerase IIα. Red dashed lines represent coordinate bond interactions with Mg$^{2+}$. Red tiny, dashed lines are hydrogen bonding interactions with amino acid Asp 543, and Asp 831. Mg$^{2+}$ is shown as a nonbonded sphere (crimson red). Residues that are involved in hydrogen bonding are shown in the stick presentation.

Compound 6 interacted in a similar pattern, the oxygen atoms of both coplanar carbonyl groups at positions 3 and 4 formed coordinate bond interactions with Mg$^{2+}$ and H-bond interaction with amino acid Asp 543. Moreover, compound 6 interacted as an H-bond acceptor through the carbonyl group of its pyrazolidine moiety with amino acid Asp 831 (Figure 19). Regarding compound
8a, it interacted with the Topo II enzyme through coordinate bonding with the magnesium ion Mg$^{2+}$ via the oxygen atoms of both carbonyl groups at positions 3 and 4 and H-bond interaction with amino acid Asp 543. Additionally, it interacted as an H-bond acceptor with amino acids His 759 and Gly 725 through the N atom of piperazine and carbonyl group of indoline moiety, respectively. It exerted arene cation interaction with Arg 713 (Figure 20).

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