Novel sulphonamide-bearing methoxyquinazolinone derivatives as anticancer and apoptosis inducers: synthesis, biological evaluation and in silico studies

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ABSTRACT

We synthesised a new series of sulphonamide-bearing quinazolinone derivatives 5–18 and evaluated their in vitro cytotoxicity in various cancer cell lines (A549, HepG-2, LoVo and MCF-7) and in normal human cells (HUVEC). Compounds 6 and 10 exhibited the higher activity against all the cancer cell lines compared with 5-flourourcil as positive control. The ability of the most promising compounds 6 and 10 to induce cell cycle arrest and apoptosis in breast cancer (MCF-7) cells was evaluated by flow cytometry. Reverse transcriptase-polymerase chain reaction and western blotting were used to evaluate the expression of apoptosis-related markers. We found that the 2-tolythioacetamide derivative 6 and the 3-ethyl phenyl thiocetamide derivative 10 exhibited cytotoxic activity comparable to that of 5-flourouracil as reference drug in MCF-7 and LoVo colon cancer cells. Cell cycle analysis showed a concentration-dependent accumulation of cells in the sub-G1 phase upon treatment with both compounds. The Annexin V-fluorescein isothiocyanate/propidium iodide assay showed that the compounds 6 and 10 increased the early and late apoptosis cell death modes in a dose-dependent manner. These compounds downregulated the expression of B-cell lymphoma-2 (Bcl-2), while increasing that of p53, Bcl-2-like protein 4, and caspase-7, at the mRNA and protein levels. Molecular docking of compounds 6 and 10 with Bcl-2 predicted them to show moderate—high binding affinity (6: –7.5 kcal/mol, 10: –7.9 kcal/mol) and interactions with key central substrate cavity residues. Overall, compounds 6 and 10 were found to be promising anticancer and apoptosis-inducing agents.

1. Introduction

Cancer is a life-threatening disease that is considered a major medical challenge worldwide\textsuperscript{1}. Treatments for cancer include surgery, chemotherapy, hormonal therapy, and biological therapy\textsuperscript{2–4}. The choice of treatment is influenced by the site and progression of the disease. Chemotherapy is primarily used for the treatment of metastasis and hypoxic tumours. However, its use is limited by the toxicity of the drugs towards healthy cells\textsuperscript{5,6}. This toxicity is a result of the low selectivity of existing chemotherapeutic drugs towards cancer cells\textsuperscript{7}. In addition, the long-term use of chemotherapeutic agents often gives rise to drug resistance\textsuperscript{8}. The continuous search for new anticancer agents that offer both selectivity towards malignant cells and low potential for resistance is required. Interest in quinazolinone derivatives grew after showing numerous activity in medical chemotherapy such as apoptosis induction and antiangiogenic properties. Idelalisib, afatinib, gefitinib, erlotinib, and lapatinib (Figure 1) are among these compounds that have been reported to exert apoptosis induction and cell cycle arrest in different cancer cell lines\textsuperscript{9–14}. The main factors contributing to the interest in these compounds are their good safety profile and potential for oral administration\textsuperscript{15,16}. Hybridisation has proven to be beneficial in the preparation of new anticancer agents and in overcoming the drawbacks of conventionally used drugs\textsuperscript{17,18}. Therefore, sulphonamides were hybridised with quinazolin-4(3H)-ones to obtain potentially better drug candidates. Sulphonamides mimic the properties of p-aminobenzoic acid and block SH- and NH\textsubscript{2}-containing enzymes and proteins to exhibit antimicrobial activity\textsuperscript{19,20}.

We designed a series of novel compounds using the quinazoline-sulphonamide hybrid scaffold and explored their in vitro cytotoxic effects against a range of cancer cell lines. The hybrid group was fixed and structural modifications focussed on the replacement of the thiol group at the C-2 position of quinazolines with thioacetamide derivatives bearing substituted phenyl rings. The activities of the target compounds were evaluated against MCF-7, HepG-2, LoVo, and A549 cancer cell lines; the most potent compounds were then evaluated for their pro-apoptotic activity, in order to analyse the underlying anticancer mechanisms. To explore the possible pharmacological properties involved in the anticancer activity, the cell cycle and apoptosis were evaluated by flow cytometry. The target compounds were established to initiate...
apoptosis, which synergistically enhanced the antitumor effects. Therefore, the apoptotic effects of the most potent compounds were explored by evaluating caspase-7 activity and by monitoring B-cell lymphoma-2 (Bcl-2) and Bcl-2-like protein 4 (Bax) levels using in vitro and in silico techniques.

2. Results and discussion

2.1. Chemistry

In this work, it seemed of interest to search for new heterocyclic compounds with anticancer activity. A novel series of 3,4-dihydroquinazolinone conjugated to a biologically active benzenesulphonamide moiety was synthesised by the introduction of benzenesulphonamide at the 3-position with the incorporation of different types of acetamide terminal at 2-position aimed at exploring the potential anticancer activity. Scheme 1 shows the synthesis of quinazolinone-benzenesulphonamide derivatives 5–18. The starting material, 4-(2-mercapto-4-oxoquinazolin-3(4H)-yl) benzenesulphonamide (4) was prepared in quantitative yield by cyclocondensation of 4-isothiocyanatobenzensulfonamide (2) and 2-amino-3-methoxybenzoic acid (3) in refluxing 1,4-dioxan containing a catalytic amount of triethylamine. The structure of compound 4 was characterised from correct analytical data as well as its infra-red (IR) spectrum which showed characteristic bands at 3321, 3262, 3181 cm\(^{-1}\) (NH\(_2\)), 3055 cm\(^{-1}\) (CH aromatic), 1691 cm\(^{-1}\) (CO), 1620 cm\(^{-1}\) (CN), 1381, 1156 cm\(^{-1}\) (SO\(_2\)). \(^1\)H-NMR spectrum exhibited signals at 3.9 ppm attributed to OCH\(_3\) and 12.3 ppm assigned to SH group. \(^13\)C-NMR spectrum revealed signals at 55.7 ppm due to OCH\(_3\), 160.5 ppm for C=\(\equiv\)N and 160.9 ppm attributed to C=O. The coupling of 4 and 2-chloro-N-substituted acetamides in dry acetone, in the presence of anhydrous K\(_2\)CO\(_3\) at room temperature yielded the corresponding 2-((8-methoxy-4-oxo-3-(4-sulfamoylphenyl)-3,4-dihydroquinazolin-2-yl)thio)-N-substituted phenyl acetamides 5–18. The synthesised compounds 5–18 were characterised on the basis of their spectral data. The IR spectra of compounds 5–18 displayed additional NH, NH\(_2\), CH aromatic, CH aliphatic, 2CO, CN and SO\(_2\) characteristic bands in the assigned regions. Proton nuclear magnetic resonance (\(^1\)H-NMR) spectra of compounds 5–18 revealed two signal peaks (3.9 – 4.1 ppm, representing the CH\(_2\), 7.9 – 10.3 ppm, representing the NH) and the loss of the SH singlet of 3. The \(^13\)C-NMR spectra of compounds 5–18 showed two signals peculiar to the CH\(_2\) and CO carbons. The \(^1\)H-NMR spectra of compounds 6–8 showed singlet peaks at 2.4, 2.2 and 2.3 ppm, which were attributed to the CH\(_3\) groups at the \(\textit{ortho}\), \(\textit{meta}\) and \(\textit{para}\) positions of the phenyl group, respectively. The \(^1\)H-NMR and \(^\text{13}\)C- spectra of compounds 9–11 showed triplets (1.0, 1.1, 1.1 ppm, respectively) and quartettes at 2.5 ppm, attributed to the CH\(_3\) and CH\(_2\), respectively of the ethyl groups, at the \(\textit{ortho}\), \(\textit{meta}\) and \(\textit{para}\) positions of the phenyl ring. The \(^\text{13}\)C-NMR spectra of compounds 9–11 showed signals corresponding to the CH\(_3\) (14.5, 15.9 and 16.1 ppm, respectively) and CH\(_2\) (24.0, 28.7, and 28.0, respectively) of the ethyl groups. The IR spectra of compounds 16–18 showed bands corresponding to the NO\(_2\) groups in the specified region. The \(^1\)H-NMR spectra of 16 and 17 showed singlets at 2.3 and 2.2 ppm, respectively, due to the CH\(_3\) group, while the \(^\text{13}\)C-NMR showed signals at 19.3 and 18.0 ppm, respectively.
2.2. Biological evaluation

2.2.1. Cytotoxicity screening

The anticancer activity of compounds 5–18 was evaluated against a panel of cell lines, including non-small cell lung carcinoma (A549), liver (HepG2), colon (LoVo), and breast (MCF-7) cancer cell lines, as well as normal human umbilical vein endothelial cells (HUVEC), by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. 5-Flourouracil was used as reference drug. As shown in Table 1, the screening results indicated that eight compounds, namely 3, 4-dihydroquinazolin-sulphonamide carrying 2-tolylthioacetamide group at 2-position 6, 3- tolyl 7, and...
3-ethylphenyl 10, 4-ethylphenyl 11, 4-ethoxyphenyl 13, 3,5-dimethoxyphenyl 14, 3,4,5-trimethoxyphenyl 15 and 2-methyl-4-nitrophenyl 16 exhibited powerful cytotoxic activity against all cancer cell lines compared to 5-flouroauracil as positive control. In general, all the tested compounds tended to exhibit better efficacy against MCF-7 cells than against the other cell lines. Among the compounds screened, the 2-tolyl derivative 6 and the 3-ethylphenyl derivative 10 exhibited the most promising activity. The 2-tolyl derivative 6 exhibited good antitumor activity, with half maximal inhibitory concentrations (IC50 values 20.17, 22.64, 45.57, and 51.50 µM, against MCF-7, LoVo, HepG2, and A549 cell lines, respectively. Interestingly, 2-tolyl derivative 6 was found to be much less cytotoxic towards normal HUVECs (IC50 = 88.27 µM). The 3-ethylphenyl derivative 10 also exhibited good cytotoxic potency against MCF-7 cells (IC50 = 20.91 µM), LoVo (IC50 = 22.30 µM), HepG2 (IC50 = 42.29 µM), and A549 (IC50 = 48.00 µM). The presence of the methyl group at 2-position in phenyl ring in compound 6 with IC50 value = 20.17 µM and ethyl group at 3-position in phenyl ring in compound 10 with IC50 value = 20.91 µM lead to an increase in the anticancer activity against human breast cancer cell line MCF-7 compared to the 5-flouroauracil as reference drug with IC50 value 95.63 µM. It is clear from the present data that the comparison of the IC50 for the synthesised compounds against human breast cancer cell line MCF-7 follows the order 6 > 10 > 7 > 11 > 14 > 16 > 13 > 15 > 5-flouroauracil with IC50 values 20.17, 20.91, 27.69, 29.40, 34.60, 39.90, 46.72, 74.03, 95.63, respectively. On the other hand, compounds 5, 8, 9, 12, 17 and 18 showed no activity towards all the cell lines. Based on the MTT screening results, compounds 6 and 10 were found to be the most potent against MCF-7 cells, so they were pursued for further investigation.

2.2.2. Cell cycle analysis

The capacity of anticancer drugs to influence cell cycle distribution can provide an insight into the mechanism of their cytotoxic activity. In fact, several cell cycle inhibitors have emerged as prospective therapeutic medications for the treatment of several tumours. Following the cytotoxicity screening, the effect of the most active compounds 6 and 10 on cell cycle progression in the MCF-7 cell line was evaluated. Compared with untreated cells, MCF-7 cells treated with compounds 6 and 10 had a significantly higher percentage of cells in the sub-G1 phase. This increase in sub-G1 phase cells was dose-dependent. Treatment with 10, 20, and 30 µM of the promising compound 6 increased the proportion of sub-G1 phase cells to 3.35 ± 0.07%, 7.8 ± 0.14%, and 22.85 ± 1.2%, respectively, versus the control (0.75 ± 0.07%) (Figure 2). Similarly, treatment of MCF-7 cells with the active compound 10 (10, 20, and 30 µM) also caused an accumulation of cells in the sub-G1 phase (4.3 ± 0.28%, 7.25 ± 2.89% and 30.6 ± 0.07%, respectively), compared to the control (0.75 ± 0.07%) (Figure 3). This increase in the proportion of sub-G1 phase cells was accompanied by a significant decrease in the percentage of cells in the G1 and G2-M phases. It has been proposed that the increment in

![Figure 2](image-url). Cell cycle distribution of MCF-7 cells analysed by flow cytometry after treatment with compound 6 at (10, 20 and 30 µM) concentrations for 24 h. The histogram showing the cells percentage in control and treated cells. Columns, average ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001 as compared to control.
the sub-G1 cell fraction is indicative of apoptotic cell death\textsuperscript{26}, suggesting that both the biologically active compounds \textsuperscript{6} and \textsuperscript{10} induced apoptosis in MCF-7 cells.

\subsection*{2.2.3. Quantification of apoptosis}

Apoptosis evasion is also a hallmark of the transformation of normal cells into tumour cells\textsuperscript{27}. Common anticancer drugs aim to induce cell death through apoptosis; this is viewed as a requirement for blocking malignant cell growth\textsuperscript{28}. To verify that apoptotic cell death was caused by the promising compounds \textsuperscript{6} and \textsuperscript{10}, an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay was used to quantify the cells undergoing apoptosis.

In addition to the accumulation of cells in the sub-G1 phase, the Annexin V/PI assay has been widely utilised for the detection of apoptotic cells\textsuperscript{29}. As shown in Figure 4, the treatment of MCF-7 cells with compound \textsuperscript{6} (10, 20, and 30 \textmu M) for 24 h increased the proportion of cells in early apoptosis (7.6 ± 1.4\%, 11.4 ± 1.97\%, and 18.35 ± 2.4\%, respectively), compared to the control group (2.9 ± 0.84\%). Similarly, the number of early apoptotic MCF-7 cells increased to 9.55 ± 0.2\%, 11.4 ± 0. 7\%, and 16 ± 0.84\% on 24 h-treatment with 10, 20, and 30 \textmu M compound \textsuperscript{10}, respectively, compared to the control group (3.3 ± 0.28\%). An increase in the number of late apoptotic cells was also observed (Figure 5).

Overall, the flow cytometry data suggested that both compounds induced cell death through the induction of apoptosis, in a dose-dependent manner.

\subsection*{2.2.4. Expression of p53, bax, caspase-7 and bcl-2}

Next, we explored whether the induction of apoptosis caused by the active compounds \textsuperscript{6} and \textsuperscript{10} was associated with the activation of apoptosis-related genes. Members of the Bcl-2 family, especially the pro-apoptotic Bax and anti-apoptotic Bcl-2 genes, are known to play a crucial role in the regulation of the apoptotic pathway. The Bax and Bcl-2 genes participate in the downstream initialising of caspase proteins\textsuperscript{30}. Therefore, the expression of key genes and the levels of proteins that control the apoptosis pathway were investigated to evaluate the pro-apoptotic effect of the compounds. The expression of p53, Bax, caspase-7, and Bcl-2 was evaluated using specific primers and antibodies. \textbeta -actin was used as an internal control. A remarkable change in the expression of apoptotic genes was reported after 24 h of treatment with increasing concentrations of the two compounds. The expression of p53, Bax, and caspase-7 mRNA increased as compared to the control, when the concentrations of compounds \textsuperscript{6} and \textsuperscript{10} were increased (Figure 6(A,B)). Meanwhile, the expression of Bcl-2 was
downregulated with increasing doses of compounds 6 and 10, as compared to that of the control.

To assess the changes in expression of p53, Bax, Bcl-2, and caspase-7 proteins upon treatment with the compounds, measurements of the protein levels in MCF-7 cells treated with different dosed of compounds 6 and 10 were carried out. Western blot analysis revealed that treatment with compounds 6 and 10 markedly increased the levels of p53, Bax, and caspase-7 (Figure 7(A,B)). In contrast, the expression of Bcl-2 was down-regulated; this shift in the Bax/Bcl-2 ratio corresponds with the onset of cell apoptosis31. The activation of caspase-7 was also evident (Figure 7), indicating the initiation of cellular apoptosis by both compounds.

2.3. Structure–activity relationship

By comparing the experimental cytotoxicity of the novel synthesised compounds reported in this study to their structures, the following structure–activity relationships (SAR) were postulated.

- The presence of a basic skeleton quinazolin-sulphonamide is necessary for the broad spectrum of cytotoxic activity of the most compounds namely 6, 7, 10, 11, 13–16 towards different cell lines (A 549, HepG2, LoVo and MCF-7).
- Introducing 2-tolyl at 2- position with the biologically active benzenesulphonamide moiety at 3- position and methoxy group at 8- position to the quinazoline 6 enhances the cytotoxic activity against all the cell lines.
- Also, the incorporating 3-ethylphenyl at 2- position to quinazoline-sulphonamide 10 lead to increase the cytotoxicity towards all cell lines.
- Transformation of 2-tolyl in compound 6 or 3-ethylphenyl in compound 10 to 3-tolyl in compound 7, 4-ethylphenyl in compound 11, 4-ethoxyphenyl in compound 13, 3,5-dimethoxyphenyl in compound 14, 3,4,5- trimethoxyphenyl in compound 15 and 2-methyl-4-nitrophenyl in compound 16 diminished the anticancer activity towards all the cell lines.
- The replacement of 3-ethylphenyl 10 by 4-ethylphenyl 11 lead to decrease the cytotoxic activity against A549, HepG2 and LoVo and MCF-7 cell lines.
- In addition the substitution of 3,5-dimethoxyphenyl 14 by 3,4,5-trimethoxyphenyl 15 reduces the anticancer activity towards all the cell lines.

2.4. Molecular docking analysis

Molecular docking simulations for promising compounds were also performed to obtain further understanding into differential cytotoxic action of synthesised compounds. The molecular docking procedure implemented in this study was validated as described by Al Ajmi and co-workers32. The cognate ligand, 1-(2-((3S)-3-(aminomethyl)-3,4-dihydroisoquinolin-2(1H)-yl)carbonyl)
phenyl)-4-chloro-5-methyl-N,N-diphenyl-1H-pyrazole-3-carboxamide (DRO), was extracted from the ligand-bound X-ray co-crystal structure of Bcl-2, and re-docked. The poses of the bound and re-docked ligand were compared, and the root mean square deviation (RMSD) was calculated. The RMSD of the re-docked DRO was found to be 0.3842 Å. Since the calculated RMSD was within the acceptable limit (2.0 Å), we were confident in adopting the docking protocol to predict the binding of compounds 6 and 10 with Bcl-2.

Anti-apoptotic Bcl-2 protein overexpression is frequently linked to several types of cancer. Recently, a lot of attention on developing effective inhibitors to reduce the increased levels of this protein. In this study, the molecular docking of compounds 6 and 10 with Bcl-2 was performed using Autodock 4.2, and the binding was compared with that of DRO (Table 2). We found that both compounds 6 and 10 occupied a similar position at the Bcl-2 binding site as DRO (Figures 8 and 9). Analysis of the interactions between compound 6 and Bcl-2 revealed that the
protein-ligand complex was stabilised by a hydrogen bond with Asp99, and nine hydrophobic interactions with Phe63, Phe71, Leu96, Arg105, and Ala108 (Table 2). The compound 6-Bcl-2 complex was further stabilised by van der Waals’ interactions of the compound with several other residues, including Asp70, Met74, Val92, Glu95, Arg98, Gly104, and Phe112. Similarly, compound 10-Bcl-2 complex was stabilised by three hydrogen bonds, with His79, Leu78, and Arg88, and an electrostatic interaction with Arg88. Compound 10 formed a further six hydrophobic interactions with Phe71, Met74, and Ala108 (Table 2).

Table 2. Molecular docking of compounds 6 and 10 with Bcl-2

| Donor atoms | Acceptor atom | Distance (Å) | Type of interaction | Estimated binding free energy, ΔG (kcal mol⁻¹) | Estimated binding affinity, Kd (M⁻¹) |
|------------|---------------|--------------|---------------------|-------------------------------------|----------------------------------|
| Bcl-2 and DRO | LIG: N ASP70: OD2 | 2.9553 | Conventional hydrogen bond | −10.2 | 3.03 × 10⁻⁷ |
| | ASP70: OD2 LIG | 4.8135 | Electrostatic (π-anion) | | |
| | MET74: CE LIG | 3.5674 | Hydrophobic (π-σ) | | |
| | LEU96: CD LIG | 3.8681 | Hydrophobic (π-π) | | |
| | PHE63 LIG | 5.2880 | Hydrophobic (π-π T-shaped) | | |
| | PHE71 LIG | 4.9682 | Hydrophobic (π-π T-shaped) | | |
| | LIG: C LEU96 | 4.9539 | Hydrophobic (alkyl) | | |
| | LIG ARG105 | 5.2941 | Hydrophobic (π-alkyl) | | |
| | LIG ALA108 | 4.4414 | Hydrophobic (π-alkyl) | | |
| | LIG LEU96 | 5.1764 | Hydrophobic (π-alkyl) | | |
| | LIG ALA108 | 4.4935 | Hydrophobic (π-alkyl) | | |
| | LIG VAL92 | 5.0873 | Hydrophobic (π-alkyl) | | |
| Bcl-2 and Compound 6 | LIG:H ASP99:OD2 | 2.8856 | Conventional hydrogen bond | −7.6 | 3.75 × 10⁻⁷ |
| | LIG:C PHE71 | 3.7687 | Hydrophobic (π-σ) | | |
| | A:PHE63 LIG | 5.2080 | Hydrophobic (π-π T-shaped) | | |
| | A:ALA108 LIG:C | 3.5709 | Hydrophobic (alkyl) | | |
| | LIG:C LEU96 | 4.7454 | Hydrophobic (alkyl) | | |
| | LIG:C ARG105 | 4.6973 | Hydrophobic (alkyl) | | |
| | LIG ALA108 | 4.3570 | Hydrophobic (π-alkyl) | | |
| | LIG LEU96 | 5.3899 | Hydrophobic (π-alkyl) | | |
| | LIG ARG105 | 5.2307 | Hydrophobic (π-alkyl) | | |
| | LIG ALA108 | 4.9292 | Hydrophobic (π-alkyl) | | |
| Bcl-2 and Compound 10 | HIS79: HD1 LIG: O | 2.0200 | Conventional hydrogen bond | −7.9 | 6.23 × 10⁻⁵ |
| | ARG88: HH1 LIG: S | 2.9013 | Conventional hydrogen bond | | |
| | LIG: H LEU78: O | 1.9625 | Conventional hydrogen bond | | |
| | ARG88: NH1 LIG | 3.3619 | Electrostatic (π-cation) | | |
| | MET74: CE LIG | 3.8605 | Hydrophobic (π-σ) | | |
| | LIG:C PHE71 | 3.6647 | Hydrophobic (π-σ) | | |
| | LIG:S HIS79 | 5.3102 | π-sulfur | | |
| | LIG:C MET74 | 4.9968 | Hydrophobic (alkyl) | | |
| | LIG MET74 | 4.6108 | Hydrophobic (π-alkyl) | | |
| | LIG ALA108 | 4.8136 | Hydrophobic (π-alkyl) | | |

*Chemically DRO is 1-[(1S)-3-(aminomethyl)-3,4-dihydroisoquinolin-2(1H)-yl carbonyl] phenyl]-4-chloro-5-methyl-N,N-diphenyl-1H-pyrazole-3-carboxamide. It is the cognate ligand of Bcl-2.*
The DRO-Bcl-2 complex was further stabilised by van der Waals’ interactions of DRO with the Arg88, Glu95, Gly104, and Phe112 residues of Bcl-2. The binding energies and the corresponding binding affinities of DRO, compounds 6 and 10 towards Bcl-2 were estimated to be $-10.2 \text{ kcal mol}^{-1}$ and $3.03 \times 10^7 \text{ M}^{-1}$, $-7.6 \text{ kcal mol}^{-1}$ and $3.75 \times 10^7 \text{ M}^{-1}$ and $-7.9 \text{ kcal mol}^{-1}$ and $6.23 \times 10^5 \text{ M}^{-1}$, respectively (Table 2). It is interesting to note that the Phe63, Tyr67, Phe71, Met74, Leu96, and Ala108 residues of Bcl-2 showed interactions with both compound 6 and DRO, while the Phe63, Tyr67, Phe71, Met74, Val92, Leu96, and Ala108 residues showed interactions with compound 10 and DRO.

3. Experimental
3.1. Chemistry
The melting points (MP; uncorrected) of the compounds were determined in an open capillary on a Gallen Kamp melting point apparatus (Sanyo Gallen Kamp, UK). Precoated silica gel plates (Kieselgel 0.25 mm, 60 F254, Merck, Germany) were used for thin-layer chromatography. A developing solvent system of chloroform/methanol (8:2) was used, and the spots were visualised using ultraviolet light. The IR spectra (KBr disc) were recorded using a Fourier transform-IR spectrophotometer (Perkin Elmer, USA). A nuclear magnetic resonance spectrophotometer (Bruker AXS Inc., Switzerland) was used for the $^1H$- and $^13C$-NMR experiments, operating at 500 MHz and 125.76 MHz, respectively. Chemical shifts are reported as δ-values (ppm) relative to tetramethylsilane (internal standard), using deuterated dimethyl sulfoxide (DMSO-d$_6$) as the solvent. Elemental analyses were conducted using a model 2400 CHNSO analyser (Perkin Elmer, USA). All results were within ±0.4% of the theoretical values. All reagents used were of analytical grade.

4--(2-Mercapto-8-methoxy-4-oxoquinazolin-3(4H)-yl) benzenesulphonamide (4)
A mixture of 2-amino-3-methoxybenzoic acid 3 (1.67 g, 0.01 mol), 4-isothiocyanatobenzenesulphonamide 2 (2.14 g, 0.01 mol) in dioxan (30 ml) containing a catalytic amount of triethylamine was refluxed for 8 h and then left to cool. The solid product was collected by filtration and recrystallized from dioxan to yield 4.

4: Yield: 92%. MP: 342–344 °C. IR (KBr, cm$^{-1}$): 3321, 3262 (NH$_3$), 3055 (aromatic), 1691 (CO), 1620 (CN), 1381, 1156 (SO$_2$). $^1H$-NMR (DMSO-d$_6$,ppm): 3.9 (s, 3H, OCH$_3$), 7.0–7.7 (m, 5H, Ar-H + SO$_2$NH$_2$), 7.8, 8.0 (2d, 4H, J=7.1, 7.0 Hz, AB system), 12.3 (s, 1H, SH).
3.1.1. General procedure for the synthesis of 3, 4-di-hydroquinazolin-sulphamoyl derivatives (5–18)
Anhydrous K₂CO₃ (1.38 g) was added to a mixture of 4 (0.63 g, 0.01 mol) and 2-chloro-N-substituted acetamide derivatives (0.012 mol) in dry acetonitrile (50 ml) and stirred at room temperature for 8 h. The resulting solid was then collected by crystallisation from ethanol to yield compounds 5–18.

2-((8-Methoxy-4-oxo-3-(4-sulphamoylphenyl)-3,4-dihydroquinazolin-2-yl)thio)-N-phényl-acetamide (5)
5: Yield: 77%. MP: > 350 °C. IR (KBr, cm⁻¹): 3324, 3291, 3164 (NH₂, NH), 3056 (aromatic), 2987, 2845 (aliphatic), 1687, 1663 (2 CO), 1619 (CN), 1385, 1155 (SO₂). ¹H-NMR (DMSO-d₆, ppm): 3.8 (s, 3H, OCH₃), 4.0 (s, 2H, CH₂), 7.3, 7.5 (2d, 4H, J = 7.3, 7.2 Hz, AB system), 7.6 – 8.0 (m, 10H, Ar-H + SO₂NH₂), 10.3 (s, 1H, NH). ¹³C-NMR (DMSO-d₆, ppm): 31.3, 56.6, 116.5, 117.4, 118.6 (2), 119.6, 124.8 (2), 126.9, 127.4, 129.2 (2), 130.3 (2), 130.7 (2), 134.1, 143.9, 151.3, 157.8, 159.2, 175.6. MS (% m/z): 496 (M⁺) (32.21), 77 (100). Anal. Calcd. for C₂₅H₂₄N₄O₅S₂ (524.60): C, 55.63; H, 4.06; N, 11.28. Found: C, 55.87; H, 4.19; N, 11.49.

N-(2-Ethylphenyl)-2-((8-methoxy-4-oxo-3-(4-sulphamoylphenyl)-3,4-dihydroquinazolin-2-yl)thio) acetamide (9)
9: Yield: 92%. MP: 288 – 290 °C. IR (KBr, cm⁻¹): 3412, 3387, 3178 (NH₂, NH), 3100 (aromatic), 2988, 2827 (aliphatic), 1685, 1666 (2 CO), 1612 (CN), 1391, 1155 (SO₂). ¹H-NMR (DMSO-d₆, ppm): 1.0 (t, 3H, J = 6.6 Hz, CH₃ ethyl), 2.5 (q, 2H, J = 8.3 Hz, CH₂ ethyl), 3.8 (s, 3H, OCH₃), 4.1 (s, 2H, CH₂), 7.7, 7.8 (2d, 4H, J = 6.9, 6.8 Hz, AB system), 7.1 – 7.6 (m, 9H, Ar-H + SO₂NH₂), 9.6 (s, 1H, NH). ¹³C-NMR (DMSO-d₆, ppm): 14.5, 24.0, 31.0, 56.7, 116.3, 118.1, 120.9, 126.4 (2), 126.5, 127.1, 127.5, 128.9 (2), 130.7 (2), 135.2 (2), 138.0, 138.5, 139.0, 153.7, 155.5, 161.0, 166.5. MS (% m/z): 524 (M⁺) (54.93), 121 (100). Anal. Calcd. for C₂₅H₂₅N₂O₅S₂ (524.61): C, 57.24; H, 4.61; N, 10.68. Found: C, 57.51; H, 4.86; N, 10.46.

N-(3-Ethylphenyl)-2-((8-methoxy-4-oxo-3-(4-sulphamoylphenyl)-3,4-dihydroquinazolin-2-yl)thio) acetamide (10)
10: Yield: 86%. MP: 292 – 294 °C. IR (KBr, cm⁻¹): 3345, 3300, 3152 (NH₂, NH), 3078 (aromatic), 2945, 2856 (aliphatic), 1689, 1661 (2 CO), 1618 (CN), 1390, 1165 (SO₂). ¹H-NMR (DMSO-d₆, ppm): 1.1 (t, 3H, J = 6.5 Hz, CH₃ ethyl), 2.5 (q, 2H, J = 6.8 Hz, CH₂ ethyl), 3.9 (s, 3H, OCH₃), 4.0 (s, 2H, CH₂), 7.9, 8.0 (2d, 4H, J = 7.0, 7.1 Hz, AB system), 7.2 – 7.7 (m, 9H, Ar-H + SO₂NH₂), 10.2 (s, 1H, NH). ¹³C-NMR (DMSO-d₆, ppm): 15.9, 28.7 (2), 56.7, 117.0, 118.1, 118.9, 120.9, 123.4, 126.9 (2), 127.1, 127.4, 129.1, 130.3 (2), 130.7, 138.0, 139.1, 139.3, 144.7, 153.7, 155.6, 161.0, 166.0. MS (% m/z): 524 (M⁺) (22.76), 231 (100). Anal. Calcd. for C₂₅H₂₅N₂O₅S₂ (524.61): C, 57.24; H, 4.61; N, 10.68. Found: C, 57.02; H, 4.35; N, 10.89.

N-(4-Methylphenyl)-2-((8-methoxy-4-oxo-3-(4-sulphamoylphenyl)-3,4-dihydroquinazolin-2-yl)thio) acetamide (11)
11: Yield: 88%. MP: 280 – 282 °C. IR (KBr, cm⁻¹): 3400, 3363, 3145 (NH₂, NH), 3075 (aromatic), 2945, 2867 (aliphatic), 1687, 1665 (2 CO), 1619 (CN), 1378, 1163 (SO₂). ¹H-NMR (DMSO-d₆, ppm): 1.1 (t, 3H, J = 8.4 Hz, CH₃ ethyl), 2.5 (q, 2H, J = 7.9 Hz, CH₂ ethyl), 3.8 (s, 3H, OCH₃), 4.0 (s, 2H, CH₂), 7.1, 7.4 (2d, 4H, J = 7.6, 7.7 Hz, AB system), 7.7 – 8.0 (2d, 4H, J = 7.4, 7.5 Hz, AB system), 7.3 – 7.7 (m, 5H, Ar-H + SO₂NH₂), 10.3 (s, 1H, NH). ¹³C-NMR (DMSO-d₆, ppm): 16.1, 28.0 (2), 56.7, 116.4, 118.1, 119.7 (2), 120.9, 127.4 (2), 128.4 (2), 130.7 (3), 137.0, 138.0, 139.3, 139.4, 146.2, 153.7, 155.6, 161.0, 165.8. MS (% m/z): 524 (M⁺) (8.05), 106 (100). Anal. Calcd. for C₂₅H₂₅N₂O₅S₂ (524.61): C, 57.24; H, 4.61; N, 10.68. Found: C, 57.49; H, 4.78; N, 10.87.

2-(8-Methoxy-4-oxo-3-(4-sulphamoylphenyl)-3,4-dihydroquinazolin-2-yl)thio)-N-(p-methoxyphenyl) acetamide (12)
12: Yield: 66%. MP: 296 – 298 °C. IR (KBr, cm⁻¹): 3445, 3343, 3129 (NH₂, NH), 3078 (aromatic), 2976, 2845 (aliphatic), 1677, 1657 (2 CO), 1621 (CN), 1376, 1176 (SO₂). ¹H-NMR (DMSO-d₆, ppm): 3.8, 3.9 (2s, 6H, 2 OCH₃), 4.0 (2s, 2H, CH₂), 6.8, 7.4 (2d, 4H, J = 6.7, 6.6 Hz, AB system), 7.6, 8.0 (2d, 4H, J = 7.2, 7.3 Hz, AB system), 7.5–7.9
(m, 5H, Ar-H + SO₂NH₂), 10.2 (s, 1H, NH). 13C-NMR (DMSO-d₆, ppm): 32.3, 55.6, 56.9, 114.3 (2), 116.4, 118.1, 120.9, 121.2 (2), 126.9 (2), 127.4, 130.3 (2), 130.7, 132.4, 138.0, 139.1, 153.7, 155.6, 155.8, 161.0, 165.5. MS (m/z, %): 526 (M⁺) (53), 165 (100). Anal. Calcld. for C₂₅H₂₄N₄O₆S₂ (526.58): C, 54.74; H, 4.21; N, 10.64. Found: C, 54.49; H, 4.00; N, 10.40.

**N-(p-Ethoxyphenyl)-2-(8-methoxy-4-oxo-3-(4-sulphamoylphenyl)-3,4-dihydroquinazolin-2-ylthio) acetamide (13)**

**3.2. Biological evaluation**

**3.2.1. Cytotoxicity assay**

The MTT assay was performed to test the antiproliferative activity of the synthesised compounds, as per the methodology described by Alqahtani and co-workers35. Briefly, A549, HepG2, LoVo, MCF-7 and HUVEC cell lines were seeded in 96-cell culture plates (5 x 10³ per well) and allowed 24h for adherence. The cells were then treated with different concentrations of each compound, and 5-fluorouracil was used as a positive control. Following the treatment period (48 h), 10 µL of MTT solution (5 mg/mL) was added to each well and the plates were incubated at 37°C for 2–4 h. Isopropanol (100 µL) acidified with 0.1 N HCl was added to solubilise the formazan products and the plate was kept on a shaker for 10 min. The optical density of each mixture was measured at 570 nm using an enzyme-linked immunosorbent assay plate reader (ELISA plate reader, Bio-Tek, USA). The concentrations of tested compounds required to inhibit cell growth by 50% (IC₅₀) were calculated using a dose-response curve. Cell survival was calculated using the following equation:

\[
\text{Cell survival} \% = \left( \frac{\text{OD of treated sample}}{\text{OD of untreated sample}} \right) \times 100
\]

**3.2.2. Cell cycle analysis**

Cell cycle analysis was conducted as previously described by Alqahtani and co-workers36. Briefly, MCF-7 cells were seeded in 6-well plates and incubated for 24h before the addition of various concentrations (10, 20 and 30 µM) of compounds 6 and 10. After incubating for 24h, the cells were harvested, washed, and
resuspended in PBS. The cells were fixed with 70% ethanol at 4 °C for 4 h. The cells were then incubated with RNase (100 μg/mL) and PI (50 μg/mL) for 30 min in the dark. Flow cytometry analysis was performed using Cytomics FC 500 (Beckman Coulter, Brea, CA, USA).

3.2.3. Quantification of apoptosis by flow cytometric analysis
Apoptosis was measured using the Annexin V/PI detection kit (BioLegend, CA, USA), as per the manufacturer’s instructions. Briefly, MCF-7 cells were treated with different concentrations (10, 20 and 30 μM) of compounds 6 and 10 for 24 h. After treatment, the detached and adherent cells were collected, washed, and resuspended in 100 μL of 1X binding buffer. Then, 5 μL of Annexin V and 5 μL of PI were added to the resuspended cells. After incubating for 15 min in the dark, 400 μL of binding buffer was added to each tube and cell samples were analysed using Cytomics FC 500 (Beckman Coulter, Brea, CA, USA). Data collection and analysis were conducted using the CXP software V. 3.0 (Aspect, Phoenix, AZ, USA).

3.2.4. Rtpcr
MCF-7 cells were cultured with different concentrations (10, 20 and 30 μM) of compounds 6 and 10 for 24 h, and the untreated cells were used as a control. The cells were lysed using Trizol reagent (Invitrogen, USA). Total RNA was extracted as per the manufacturer’s instructions. Total RNA was quantitated by taking 2 μL of resuspended RNA on a Nano drop spectrophotometer (Thermo Scientific, USA) and reverse transcribing an equal amount (1 μg) of RNA to make complementary deoxyribonucleic acid (cDNA) using a Super Script VILO cDNA synthesis Kit (Invitrogen), as per the manufacturer’s protocol, in a final volume of 20 μL. The mixture was incubated at 42 °C for 1 h. The generated cDNA (2 μL) was used to assess the mRNA expression of apoptotic genes, including caspase-7, Bax, Bcl-2, and p53. The actin gene was used as an internal control. RT-PCR was performed using 5X Firepol Master Mix ready to load (Solis BioDyne, Tartu, Estonia), as per the manufacturer’s instructions. The specific primer sets used in this study are listed in Table 3. The program was run as follows: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, elongation at 72 °C for 45 s (30 cycles), and final extension at 72 °C for 10 min. The RT-PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide, and the gel was imaged on a Licor machine.

3.2.5. Western blot analysis
MCF-7 cells were treated with compounds 6 and 10 for 24 h. The cells were then harvested and washed twice with 1x PBS, by adding lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM sodium ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis (β-aminoethyl)-ether)-N,N,N’,N’-tetraacetic acid, 1% Triton X100, 1 μg/mL leupeptin, and 100 μM phenylmethylsulphonyl fluoride, to prepare the total cell extract. The lysate was cooled on ice for 1 h and clarified by centrifugation at 13000 rpm at 4 °C for 15 min. The supernatants were collected. The protein concentrations of all the samples were determined using Bradford reagent (BioRad, Hercules, CA, USA). For western blot analysis, 30 – 35 μg of the total protein was loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% bovine serum albumin in 0.1% Tween-tris-buffered saline (TBST) buffer for 2 h at room temperature, and then washed thrice with TBST. The membrane was incubated with primary antibodies for Bcl-2, p53, caspase7, Bax, and β-actin (1:200, Santa Cruz Biotechnology). To ensure equal loading, an anti-actin antibody was also used. The membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody diluted 1: 1000 for 1 h at room temperature. After incubation, the membrane was washed thrice with TBST. It was then ready for immunodetection; the membrane was incubated with enhanced chemiluminescence western blotting detection reagents (Amersham, Pharmacia Biotech Inc., Piscataway, NJ, USA) and bands were obtained on exposing to X-ray films (Amersham).

3.3. Molecular docking
The potential of the two most promising compounds 6 and 10 to inhibit Bcl-2 was evaluated in molecular docking experiments, conducted as described by Al-Shabib and co-workers. The 3D coordinates of Bcl-2 were retrieved from the PDB-RCSB databank (PDB ID: 2W3L). The X-ray crystal structure of DRO-bound Bcl-2 has previously been solved at a resolution of 2.10 Å. Prior to molecular docking, the protein was pre-processed to remove crystallographic water molecules and any other heteroatoms, add hydrogen atoms, assign proper bond order, and define rotatable bonds, as previously described by Rehman and co-workers. A network of H-bonds was created, and the energy of the protein was minimised using the Merck Molecular Force Field. A grid box of 27 × 30 × 25 Å, centred at 39 × 28 × −12 Å, with 0.375 Å spacing was defined as a conformation search space for the binding of ligands to Bcl-2. Finally, molecular docking between ligands and proteins was performed using Autodock 4.2 (Scripps Research, San Diego, CA, USA), as previously described by Rabbani and co-workers. Molecular docking was performed using the Lamarckian Genetic Algorithm and Solis and Wets local search methods. The initial torsions, positions, and orientations of the ligands were set randomly. For each docking run, a maximum of 2.5 × 10⁶ calculations was enumerated after setting a population size of 150 and a translational step of 0.2 Å. The quaternion and torsion steps were set to 5. Discovery Studio (BIOVIA, San Diego, CA, USA) was used to analyse the docking results and prepare the figures. Binding affinities of compounds 6 and 10 for Bcl-2 were determined from their respective binding energies (∆G), using the following relationship:

\[
\Delta G = -RT\ln K_d
\]

Here, R is the Boltzmann gas constant (1.987 cal mol⁻¹ K⁻¹) and T is the temperature (298K).
3.4. Statistical analysis

Statistical analysis was performed using OriginPro 8.5 software (OriginLab, Northampton, MA, USA). Data are shown as the mean ± standard deviation. Differences were analysed using Student’s t-test, and were considered statistically significant if \( p < 0.05 \). \( **p < 0.01 \), \( ***p < 0.001 \).

4. Conclusion

In conclusion, a new series of quinazoline-sulphonamide derivatives 5–18 were synthesised and evaluated in vitro for their anti-proliferative activity. Most of the prepared compounds were found to exhibit remarkable cytotoxicity in the MCF-7 breast cancer cell line. Compounds 6 and 10 were found to be the most promising. Flow cytometry data revealed that compounds 6 and 10 arrested the cell cycle of MCF-7 cells in the sub-G1 and induced apoptosis in cell death mode. Furthermore, changes in the expression of apoptosis-related markers at the gene and protein level were also indicative of apoptotic activity. The 2-tolyl derivative 6 and the 3-ethylphenyl derivative 10 downregulated the expression of B-cell lymphoma-2 (Bcl-2), while increasing that of p53, Bcl-2-like protein 4, and caspase-7, at the mRNA and protein levels. Molecular docking of compounds 6 and 10 also suggested that they possess good binding affinity for Bcl-2. Overall, the findings suggest that compounds 6 and 10 both possess promising anti-proliferative activity. These molecules may be further modified to develop more selective, clinically useful analogues.

Disclosure statement

The authors declare no conflicts of interest.

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