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Synthesis and Evaluation of Novel Piperazine Hybrids as Promising Anticancer Agents

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This study aimed to synthesise a novel series of piperazine analogues (**3a-k**) having anticancer potential against MCF-7, MDA-MB-231 and HeLa cell lines. Structure of the synthesised analogues were confirmed through NMR and biological evaluation were done through MTT assay taking doxorubicin as a reference standard. The synthesised derivatives exhibited appreciable cytotoxicity at low concentrations. Molecular docking examined their binding interactions with estrogen receptor-α (ERα) and epidermal growth factor receptor (EGFR), associated with MCF-7 and MDA-MB-231 cell lines, respectively, providing structural insight into their anticancer potential. The results revealed that the synthesised derivative **3h** has potential binding affinity hence blocking the activity. Therefore, the results obtained from *in vitro* and *in silico* studies identify the synthesised piperazine derivatives as promising lead compounds for further anticancer optimization. The IC_{50} values support this observation, with compounds **3g**, **3h** and **3i** exhibiting the highest cytotoxic activity among the other derivatives evaluated.

Keywords: Hybrid drug design, Piperazine derivatives, Breast cancer, Cytotoxicity, Molecular docking.

INTRODUCTION

Piperazine is a privileged heterocyclic scaffold that occupies a prominent position in medicinal chemistry due to its structural flexibility and broad pharmacological profile [1]. Its isosteric nature permits replacement of other amine-containing moieties while facilitating structural modifications that improve receptor affinity, aqueous solubility, metabolic stability and bioavailability. Owing to these advantages, piperazine has been incorporated into numerous clinically approved drugs. Several anticancer drugs, such as palbociclib [2], venetoclax [3], ribociclib [4], abemaciclib [4], brigatinib [5,6], gilteritinib [7], entrectinib [8], infigratinib [9] and trilaciclib [10], contain the piperazine framework as an essential structural element. Structural variants such as *N*-methyl piperazine, *N*-ethyl piperazine and chlorophenyl piperazine have likewise contributed to the development of kinase inhibitors and central nervous system therapeutics [11,12].

Hybrid pharmacophore design has become an effective strategy for the discovery of novel anticancer agents [13]. This approach combines complementary pharmacophoric units within a single molecular framework to enhance biological activity and optimize drug-like properties. Cyclin-dependent kinase inhibitors such as trilaciclib, palbociclib and ribociclib share the 5-(piperazin-1-yl)pyridin-2-amine scaffold linked with pyrimidine-based motifs (Fig. 1) [14]. An alternative strategy involves connecting two bioactive molecules through cleavable or non-cleavable linkers to regulate drug release under physiological or enzymatic conditions [15-17]. The present work adopts the pharmacophore hybridisation approach to construct structurally distinct piperazine-based molecules.

Breast cancer remains one of the leading causes of cancer-related mortality among women and multidrug resistance continues to compromise the effectiveness of chemotherapy [18-22]. The development of structurally novel molecules capable of overcoming these limitations remains an important objective in anticancer drug discovery.

Another pharmacologically important scaffold as selected in this study is 1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropane-1-carboxylic acid, which is present in clinically relevant drugs such as lumacaftor [23] and tezacaftor [24] (Fig. 2). This structural motif is associated with favourable physico-

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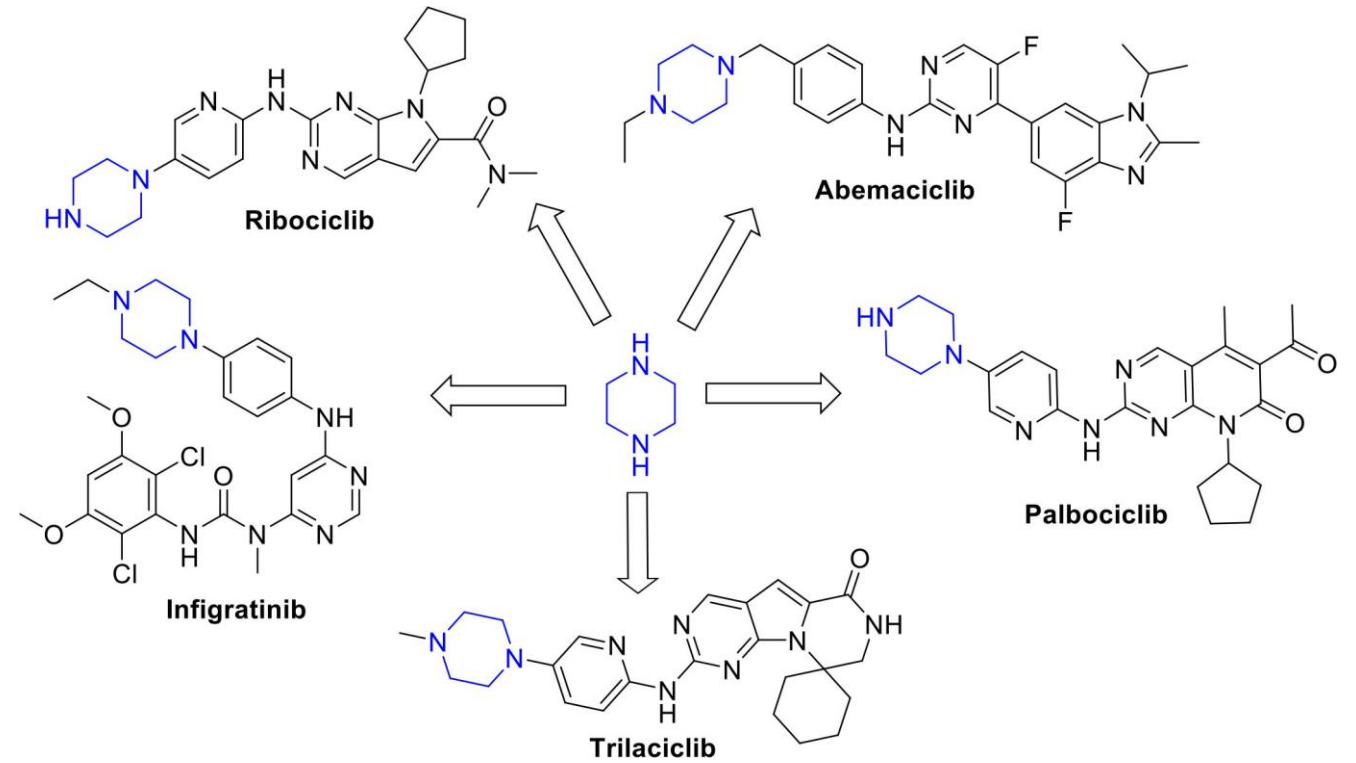


Fig. 1. Various anti-cancerous drugs derived from piperazine moiety

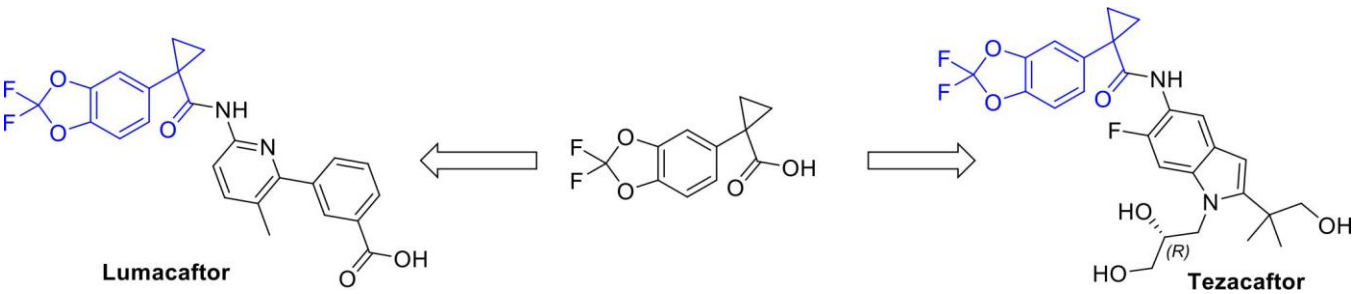


Fig. 2. Presence of 1-(2,2-difluorobenzo[*d*][1,3] dioxol-5-yl)cyclopropane-1-carboxylic acid moiety in the drugs used for the treatment of cystic fibrosis

chemical characteristics, particularly improved lipophilicity, metabolic stability and efficient target recognition, making it an attractive building block for hybrid molecule design.

Based on these considerations, a series of novel piperazine derivatives incorporating the 1-(2,2-difluorobenzo[*d*][1,3]-dioxol-5-yl)cyclopropane-1-carboxylic acid scaffold was designed and synthesized as potential anticancer agents. Although piperazine derivatives have been widely investigated in the anticancer research, the novelty of the present work lies in the integration of these two biologically relevant scaffolds into a single molecular framework. This hybrid design combines two biologically relevant pharmacophores in a single molecular framework to explore their collective contribution toward improved anticancer activity.

EXPERIMENTAL

Chemical and solvents used were purchased from BLD Pharma, Spectrochem, and Merck Ltd., India. All the reagents were of laboratory grade. Thin-layer chromatography (TLC) was performed on silica gel 60 GF_{254} (E-Merck) plates and visualised using UV light or iodine. The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$/DMSO-d_6 with a Varian Mercury plus 400/500 MHz and 100/125 MHz instruments. All the chemical shifts were reported in δ (ppm) using TMS as an internal standard. The mass spectra were recorded on Agilent ion trap MS. Meting points were recorded using Buchi melting point apparatus (M-565) and are uncorrected.

Synthesis of substituted piperazine derivatives: Initially, 1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropane-1-carboxylic acid (**1**) was allowed to react with substituted piperazines (**2a-k**) using coupling agent HATU and triethyl amine as a base in DMF as solvent. Product formation was checked after continuous stirring of 2-5 h, through TLC using DCM:MeOH (9:1) solvent system. Upon completion of the reaction, water was added and the product was extracted with ethyl acetate. The organic layer was washed successively with saturated aqueous $NaHCO_3$ and 10% NaCl solution, then concentrated under reduced pressure at 40-50 ºC. The resulting crude product was purified either by trituration with

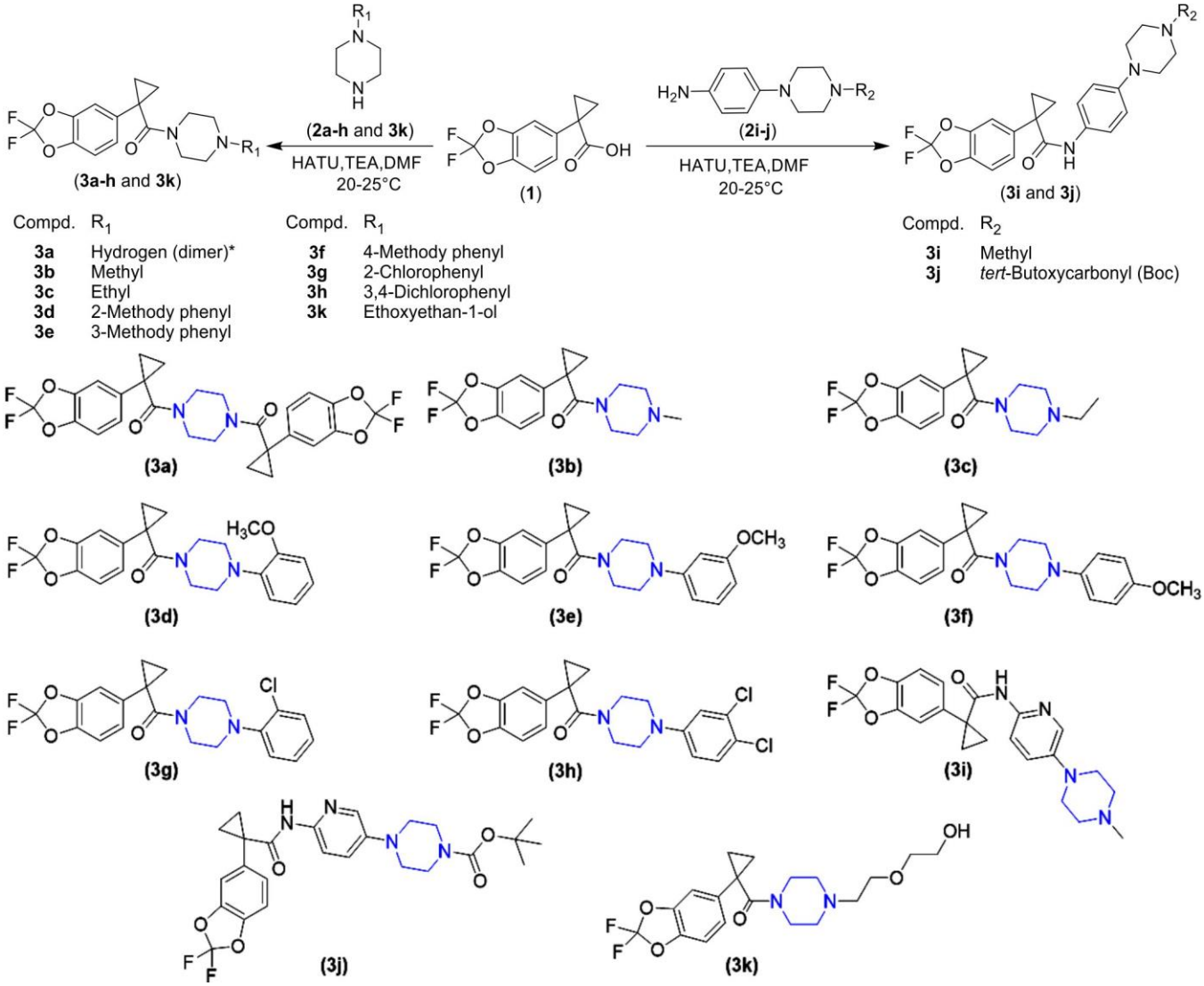
MTBE/*n*-heptane or by silica gel column chromatography (100-200 mesh) using DCM as the eluent to afford the desired piperazine derivatives of 1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropane-1-carboxylic acid (**3a-k**) in good yields (80-90%) (**Scheme-I**).

Piperazine-1,4-diyl*bis*((1-(2,2-difluorobenzo[*d*][1,3]-dioxol-5-yl)cyclopropyl)methanone) (3a): White solid; yield: 90.26%, m.p.: 189.3 ºC; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 7.00-6.94 (m, 2H), 6.88 (d, *J* = 7.6 Hz, 4H), 3.16 (d, 8H), 1.44-1.33 (m, 4H), 1.13 (q, *J* = 4.9 Hz, 4H); ^{13}C NMR (125 MHz, $CHCl_3$-d_6, δ ppm): 170.6, 144.3, 142.5, 136.7, 131.72 (t, *J* = 255.9 Hz), 120.9, 109.7, 107.3,45.3, 41.9, 29.5, 15.3; HRMS of $[C_{26}H_{22}F_4N_2O_6 + H]^+$ (*m/z*): 535.1488; calcd.: 534.1414.

(1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)-(4-methylpiperazin-1-yl)methanone (3b): Off white; yield: 83.63%, m.p.: 162.2 ºC; ^{1}H NMR (500 MHz, $CHCl_3$-d_6, δ ppm): 6.99 (d, *J* = 8.8 Hz, 1H), 6.95-6.89 (m, 2H), 3.64 (s, 2H), 3.48 (s, 2H), 2.24 (s, 7H), 1.44-1.40 (m, 2H), 1.14 (d, *J* = 7.1 Hz, 2H); ^{13}C NMR (125 MHz, $CHCl_3$-d_6, δ ppm): 170.3, 144.3, 142.4, 137.2, 131.73 (t, *J* = 255.3 Hz), 120.9, 109.5, 107.1, 54.6, 46.8, 46.0, 38.7, 29.5, 15.3, 8.9; HRMS of $[C_{16}H_{18}F_2N_2O_3 + H]^+$ (*m/z*): 325.1347; calcd.: 324.1285.

(1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)-(4-ethylpiperazin-1-yl)methanone (3c): Yellow oily mass, yield: 84.46%; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 6.97 (d, *J* = 8.8 Hz, 1H), 6.90 (dq, *J* = 3.4, 1.8 Hz, 2H), 3.63 (s, 2H), 3.47 (s, 2H), 2.36 (q, *J* = 7.2 Hz, 6H), 1.44-1.37 (m, 2H), 1.15-1.09 (m, 2H), 1.04 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, $CHCl_3$-d_6, δ ppm): 170.3, 144.3, 142.4, 137.2, 131.77 (t, *J* = 255.3 Hz), 125.4, 120.9, 109.6, 107.2, 52.4, 47.2, 29.5, 16.1, 15.4, 11.9, 8.8; HRMS of $[C_{17}H_{20}F_2N_2O_3 + H]^+$ (*m/z*): 339.1503; calcd.: 338.1442.

(1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)-(4-(2-methoxyphenyl)piperazin-1-yl)methanone (3d): Yellow oily, yield: 81.42%; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 7.06-6.80 (m, 7H), 3.85 (s, 3H), 3.64 (s, 4H), 2.95 (d,, 2H), 2.80 (s, 2H), 1.46 (q, *J* = 4.8 Hz, 2H), 1.16 (q, *J* = 4.8 Hz, 2H; ^{13}C NMR (100 MHz, $CHCl_3$-d_6, δ ppm): 170.4, 152.3, 144.3, 142.4, 140.6, 137.2, 131.75 (t, *J* = 255.6 Hz), 123.7,



Scheme-I: Synthesis of the novel piperazine hybrid drugs (**3a-k**) from the carboxylic acid (**1**) as a starting material

121.1, 121.0, 118.5, 111.4, 109.6, 107.1, 55.5, 50.5, 29.5, 15.5, 15.4; HRMS of $[C_{22}H_{22}F_2N_2O_4 + H]^+$ (*m/z*): 417.1605; calcd.: 416.1548.

(1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)-(4-(3-methoxyphenyl)piperazin-1-yl)methanone (3e): Brown semi-solid, yield: 84.91%; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 7.16 (t, 1H), 6.96 (m, 3H), 6.44 (m, 3H), 3.70 (m, 7H), 3.02 (m, 4H), 1.44 (m, 2H), 1.17 (m, 2H); ^{13}C NMR (100 MHz, $CHCl_3$-d_6, δ ppm): 170.5, 160.7, 152.3, 144.4, 142.5, 137.1, 131.77 (t, *J* = 255.5 Hz), 130.1, 121.0, 109.7, 109.4, 107.2, 105.3, 103.2, 55.3, 49.3, 29.6, 15.3; HRMS of $[C_{22}H_{22}F_2N_2O_4 + H]^+$ (*m/z*): 417.1605; calcd.: 416.1548.

(1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)-(4-(4-methoxyphenyl)piperazin-1-yl)methanone (3f): Light brown, yield: 86.07%, m.p.: 91.4 ºC; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 7.00-6.92 (m, 3H), 6.86-6.80 (m, 4H), 3.76 (s, 7H), 3.03-2.77 (m, 4H), 1.48-1.42 (m, 2H), 1.19-1.13 (m, 2H); ^{13}C NMR (100 MHz, $CHCl_3$-d_6, δ ppm): 170.4, 154.5, 145.2, 144.3, 142.4, 137.2, 131.76 (t, *J* = 255.4 Hz), 121.0, 118.9, 114.6, 109.6, 107.1, 55.7, 50.8, 29.6, 15.5, 15.3; HRMS of $[C_{22}H_{22}F_2N_2O_4 + H]^+$ (*m/z*): 417.1604; calcd.: 416.1548.

(4-(2-Chlorophenyl)piperazin-1-yl)(1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)methanone (3g): Off white, yield: 86.32%, m.p.: 92.9 ºC; ^{1}H NMR (500 MHz, $CHCl_3$-d_6, δ ppm): 7.35 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.21 (td, *J* = 7.7, 1.6 Hz, 1H), 7.01-6.93 (m, 5H), 3.73 (d, 4H), 2.89 (d, 4H), 1.48-1.44 (m, 2H), 1.20-1.14 (m, 2H); ^{13}C NMR (125 MHz, $CHCl_3$-d_6, δ ppm): 170.5, 148.6, 144.3, 142.4, 137.2, 131.74 (t, *J* = 255.3 Hz), 129.0,127.7, 124.4, 121.0, 120.6, 109.6, 107.2, 51.1, 46.2, 29.6, 15.3; HRMS of $[C_{21}H_{19}ClF_2N_2O_3 + H]^+$ (*m/z*): 421.1115; calcd.: 420.1052.

(4-(3,4-Dichlorophenyl)piperazin-1-yl)(1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)methanone (3h): Off white, yield: 87.24%, m.p.: 107.4 ºC; ^{1}H NMR (500 MHz, $CHCl_3$-d_6, δ ppm): 7.28 (s, 1H), 7.02-6.88 (m, 4H), 6.68 (dd, *J* = 8.9, 2.9 Hz, 1H), 3.69 (s, 4H), 3.01 (d, 4H), 1.44 (t, *J* = 3.5 Hz, 2H), 1.20-1.14 (m, 2H); ^{13}C NMR (125 MHz, $CHCl_3$-d_6, δ ppm): 170.5, 150.2, 144.4, 142.5, 136.9, 133.0, 131.73 (t, *J* = 255.7 Hz), 130.7, 123.3, 121.0, 117.9, 115.9, 109.7, 107.2, 48.8, 29.5, 15.2, 1.1; HRMS of $[C_{21}H_{18}Cl_2F_2N_2O_3 + H]^+$ (*m/z*): 455.0720; calcd.: 454.0663.

1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)-*N*-(5-(4-methylpiperazin-1-yl)pyridin-2-yl)cyclopropane-1-carboxamide (3i): Brown, yield: 83.36%, m.p.: 140.2 ºC; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 7.99 (d, *J* = 9.1 Hz, 1H), 7.76 (d, *J* = 3.0 Hz, 1H), 7.46 (s, 1H), 7.22-7.12 (m, 3H), 7.02 (d, *J* = 8.1 Hz, 1H), 3.08 (t, *J* = 5.1 Hz, 4H), 2.51 (t, *J* = 5.0 Hz, 4H), 2.28 (s, 3H), 1.66 (q, *J* = 3.9 Hz, 2H), 1.08 (q, *J* = 3.9 Hz, 2H); ^{13}C NMR (100 MHz, $CHCl_3$-d_6, δ ppm): 170.2, 143.3, 143.1, 143.0, 142.6, 134.6, 134.1, 130.65 (t, *J* = 256.4 Hz), 125.6, 124.8, 112.9, 111.3, 109.1, 76.3, 75.7, 53.7, 48.0, 45.0, 30.0, 15.9; HRMS of $[C_{21}H_{22}F_2N_4O_3 + H]^+$ (*m/z*): 417.1725; calcd.: 416.1660.

***tert*-Butyl4-(6-(1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)-cyclopropane-1-carboxamido)pyridin-3-yl)piperazine-1-carboxylate (3j):** Off white, yield: 81.93%, m.p.: 196.3 ºC; ^{1}H NMR (400 MHz, $CHCl_3$-d_6, δ ppm): 9.27 (s, 1H), 8.20-7.94 (m, 1H), 7.88-7.69 (m, 1H), 7.25 (dd, *J* = 9.1, 3.0 Hz, 1H), 5.18 (s, 1H), 4.26 (d, *J* = 9.8 Hz, 1H), 3.52 (t, *J* = 5.1 Hz, 4H), 3.03 (t, *J* = 5.2 Hz, 4H), 1.42 (s, 9H), 1.29-1.03 (m, 4H); ^{13}C NMR (100 MHz, $CHCl_3$-d_6, δ ppm): 171.98, 153.61, 143.37, 134.65, 126.16, 113.55, 79.15, 68.87, 48.39, 41.28, 33.18, 30.96, 27.39, 25.66-25.13 (m); HRMS of $[C_{25}H_{28}F_2N_4O_5 + H]^+$ (*m/z*): 503.2090; calcd.: 502.2028.

(1-(2,2-Difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropyl)-(4-(2-(2-hydroxyethoxy)ethyl)piperazin-1-yl)methanone (3k): Light yellow oily mass; ^{1}H NMR (500 MHz, $CHCl_3$-d_6, δ ppm): 6.96 (d, *J* = 8.9 Hz, 1H), 6.89 (dd, *J* = 6.0, 1.9 Hz, 2H), 3.69-3.63 (m, 3H), 3.58 (dt, 5H), 3.47 (d, 2H), 2.56-2.50 (m, 2H), 2.46 (s, 3H), 2.26 (s, 2H), 1.41-1.37 (m, 2H), 1.15-1.08 (m, 2H); ^{13}C NMR (125 MHz, $CHCl_3$-d_6, δ ppm): 170.3, 144.3, 142.4, 137.1, 131.7 (t, *J* = 255.3 Hz). 120.9, 109.6, 107.2, 72.5, 67.6, 62.0, 57.8, 52.9, 29.5, 15.3, 15.3; HRMS of $[C_{19}H_{24}F_2N_2O_5 + H]^+$ (*m/z*): 399.1706; calcd.: 398.1653.

Biological evaluation

***In vitro* cytotoxicity assay:** The cytotoxicity of the synthesised compounds **3a-k** were screened against breast cancer cells (MCF7, MDA-MB-231) and cervical cancer cell lines (HeLa) by performing MTT assay [25]. The protocol of the MTT assay started by routine trypsinisation step, followed by the trypsinisation and cells counting using the Hemocytometer (Improved Neubauer). Approximately 1×10^4 cells were seeded along with 100µL of complete media (DMEM+ 10% FBS+ 1% antibiotics (penicillin/streptomycin)) in each well of the flat-bottom 96-well plate. Then the well plate was subjected to a CO_2 incubator for a 24 h incubation period at 37 ºC and at 5% CO_2 to ensure cell adhesion. Upon completion of the incubation period, the media within each well was replaced with the fresh complete media prior to drug/compound treatment. The cells were treated with the drug/compound at concentrations ranging from 0 to 35 µM and the same concentration range was maintained for the positive control, which consisted of wells treated with doxorubicin. Some wells were treated with DMSO which served as the solvent control. The 96-well plate was incubated at 37 ºC under 5% CO_2 for 48 h. Cell viability for each compound were recorded using MTT assay of treated well at the 48 h of incubation. After the incubation period, the old compound containing media were replaced with 100 µL of MTT solution (90 µL of DMEM media without FBS and antibiotic and 10 µL of MTT reagent (5 mg/mL). The concentration of the MTT reagent was set to 0.5 mg/mL per plate and incubated for one more hour, then after the formation of formazan crystals inside the cell was confirmed using an inverted phase contrast microscope. Upon formation of the formazan crystals, the MTT solution was replaced with 100 µL of DMSO solution. The crystals were then dissolved by repeated shaking of the plate in a microwell plate reader and the absorbance was recorded at 570 nm. The absorbance data from the compound/drug-treated wells were normalised to the DMSO control wells. Further, the cell viability values were obtained using the below mentioned formula:

$$\text{Cell viability (\%)} = \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

Molecular docking: Molecular docking studies were conducted to study the binding interactions of new piperazine

hybrids (compounds **3g**, **3h** and **3i**) towards two relevant proteins associated with MCF-7 and the MDA-MB-231 breast cancer cell line using AutoDock Vina and structures visualised with Discovery Studio [26]. The selected protein targets were ERα (PDB ID: 1GWR) and EGFR (PDB ID: 1M17), which are clinically relevant proteins associated with hormone responsive and triple-negative breast cancer, respectively [27, 28]. ERα plays a central role in the proliferation of MCF-7 cells; however, this cell line is also regulated by several additional pathways, including PI3K/AKT/mTOR, MAPK/ERK, cyclin-dependent kinase and apoptosis-related signalling networks. Likewise, although EGFR is frequently implicated in MDA-MB-231 progression, this triple-negative breast cancer model involves numerous interconnected pathways such as PI3K/AKT, NF-κB, JAK/STAT, Wnt/β-catenin, TGF-β and epithelial-mesenchymal transition signalling. Therefore, ERβ and EGFR were selected as representative molecular targets to gain preliminary insights into potential protein–ligand interactions rather than to suggest that the observed biological activity is mediated exclusively through these pathways.

RESULTS AND DISCUSSION

A novel piperazine derivatives (**3a-k**) were synthesised through HATU-mediated amide coupling between 1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropane-1-carboxylic acid and substituted piperazines under mild reaction conditions and characterised. The protocol furnished the desired amide derivatives in high yields (80-90%) with straightforward purification, providing an efficient and versatile route for the synthesis of structurally diverse piperazine analogues suitable for biological evaluation.

Molecular docking studies: Docking results demonstrated the molecular interactions between the ligands (**3g**, **3h**, **3i**) and reference drug doxorubicin with ERα (1GWR) and EGFR (1M17) are shown in Table-1. Docking results for compounds **3g**, **3h** and **3i** exhibited favourable interactions with both ERα and EGFR proteins, as shown in Fig. 3. Among the synthesised derivatives, compound **3h** showed the most favourable docking score, with binding energies of -6.3 kcal/mol against ERα and -6.9 kcal/ mol against EGFR. The calculated binding affinities fall within the moderate range, yet they reflect favourable interactions between the compound and the binding pockets of the selected targets. These docking results are consistent with the *in vitro* cytotoxicity data and provide a structural basis for the observed biological activity.

These findings suggest that ligand **3h** exhibited the strongest binding affinity among the synthesised derivatives and may represent a promising lead for further investigation. This may be due to the chloro-substituted phenyl piperazine in the molecule that engaged in stronger hydrophobic and π-π stacking interactions with the binding pockets of the proteins. This outstanding binding affinity of **3h**, likely results from the chloro-substituted structure that may enhance lipophilicity and enable strong interactions with hydrophobic residues in the binding sites of both ERα and EGFR, similar to the studies by Al-Ghorbani *et al.* [29] who reported that piperazine derivatives with electrons withdrawing group (*i.e.* chloro) exhibited increased binding to kinase-related proteins through improved van der Waals and electrostatic interactions.

For ERα, **3h** may form hydrogen bonds with critical residues loke. Arg394, Glu353, that have been identified for ERα antagonists [30]. For EGFR, compound **3h** formed key interactions with residues such as Leu694 and Thr766, which may account for its superior binding affinity, consistent with the reported binding modes of known EGFR inhibitors [31]. Compounds **3g** and **3i** exhibited comparatively lower binding affinities but performed better than the remaining derivatives. The aminopyridine moiety in compound **3i** may enhance its affinity toward EGFR through favourable hydrogen-bonding interactions with polar residues within the active site, in agreement with the established role of nitrogen-containing heterocycles in EGFR-targeted ligand design [32]. The lower binding affinity of compound **3i** for ERα corresponds well with its weaker cytotoxic activity against MCF-7 cells. Its experimental IC_{50} value (40 ± 2.5 μM) points to preferential activity against the MDA-MB-231 cell line.

Cytotoxicity activity: Among the synthesised compounds, **3g**, **3h** and **3i** showed promising activity at micromolar concentrations. Among the synthesised derivatives, compounds **3g** and **3h** demonstrated the highest cytotoxic activity against HeLa cells within the tested series. However, direct comparisons with doxorubicin should be interpreted cautiously in the absence of statistical significance testing (Table-2). Although compound **3h** exhibited substantial growth inhibition, doxorubicin was more potent, with a lower IC_{50} value. Therefore, compounds **3g** and **3h** should be regarded as promising lead structures rather than superior alternatives to the standard drug.

For the MCF7 cell lines, compounds **3h** and **3i** showed cell viabilities of approximately 41% in comparison to doxorubicin with 46.42% cell viability and there was less potency

TABLE-1
MOLECULAR DOCKING ANALYSIS OF SYNTHESISED COMPOUNDS (**3g-i**) AND REFERENCE COMPOUND DOXORUBICIN

Compound ID	Protein target	PDB ID	Binding free energy (kcal/mol)	Key interacting amino acids	Bond length (Å)
3g	ERα	1GWR	-5.95	GLU 353, ARG 394, LEU 387	3.50, 4.00, 4.20
	EGFR	1M17	-6.25	ASP 855, LYS 745, LEU 792	3.70, 4.10, 4.30
3h	ERα	1GWR	-6.3	GLU 353, ARG 394, PHE 404	3.60, 4.00, 4.50
	EGFR	1M17	-6.9	ASP 855, MET 793, LYS 745	3.80, 4.20, 4.40
3i	ERα	1GWR	-5.75	LEU 387, ARG 394, GLU 353	3.50, 4.10, 4.20
	EGFR	1M17	-6.2	ASP 855, LYS 745, LEU 792	3.60, 4.00, 4.30
Doxorubicin	ERα	1GWR	-5.25	GLU 353, ARG 394, LEU 387	3.80, 4.10, 4.00
	EGFR	1M17	-5.24	ASP 855, LYS 745, MET 793	3.90, 4.20, 4.30

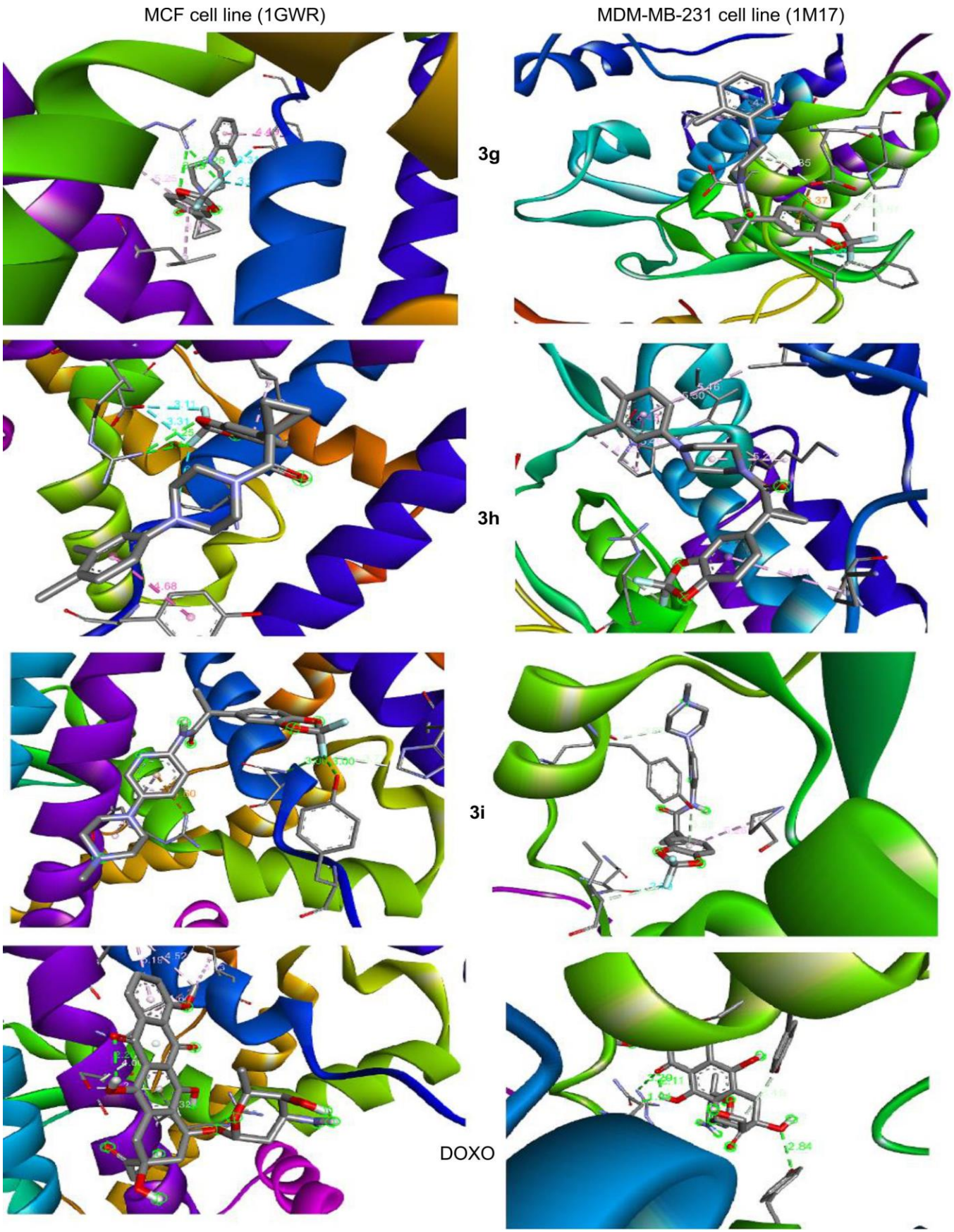


Fig. 3. Molecular docking analysis illustrating the interactions of proteins 1GWR and 1M17 from the MCF7 and MDA-MB-231 cell lines with compounds **3g**, **3h**, **3i** and the drug doxorubicin

TABLE-2
IC_{50} VALUES FOR COMPOUNDS **3g**, **3h** AND **3i**

Compounds	MDA-MB-231 cell line (μM)	HeLa cell line (μM)
3g	21 ± 8.9	20 ± 10.6
3h	27 ± 5.4	12 ± 3.7
3i	40 ± 2.5	–
Doxorubicin	14.66	7.18

against the MDA-MB-231 cell lines, with compounds with cell viabilities under 65% compared to doxorubicin with 33.74% (Fig. 4). The IC_{50} indicate that compound **3g** and **3h** possess moderate cytotoxic activity for MDA-MB-231 and HeLa cell lines, which was most likely attributed to the chloro substitution of the phenyl piperazine moiety. Compound **3i** showed IC_{50} values of 40 ± 2.5 μM against the MDA-MB-231 cell lines, which was likely due to the amino pyridine analogue. The IC_{50} results for MCF-7 cells for compounds shows limited potency might be due to poor cellular uptake or specific cell resistance mechanisms.

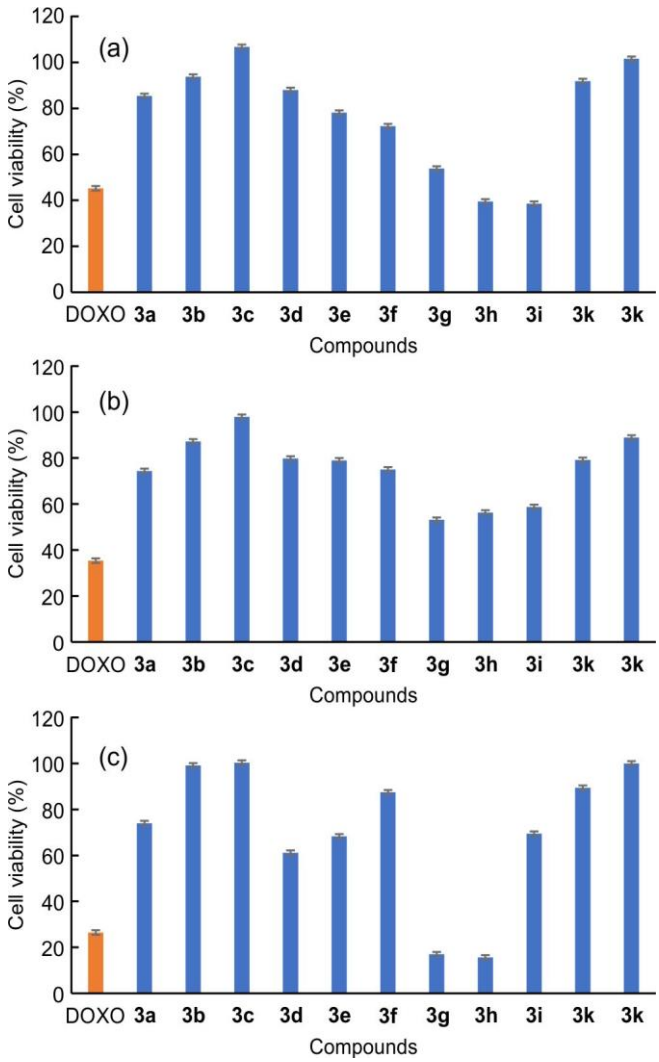


Fig. 4. Cell viability (%) of (a) MCF-7 (b) MDA-MB-231 and (c) HELA cells after exposure to 35 μM concentration against synthesised piperazine derivatives with reference drug doxorubicin (DOXO). Data are presented as mean ± SD from at least three independent experiments

The favourable activity of compounds **3g** and **3h** likely stems from a structural attribute of the chloro-substituted phenyl piperazine increasing lipophilicity and inducing better affinity for cellular targets. This supports earlier work by Rao *et al.* [33], who reported that piperazines with aryl substitutions, particularly those bearing electron-withdrawing groups, exhibited improved cytotoxicity associated with enhanced binding affinity for central molecular targets in cancer cells. The electron-deficiency of the chloro-group should allow stabilisation of the interaction of the compound with the enzymes or receptors involved in the proliferation of cancer cells, such as tyrosine kinases or DNA repair proteins, based on studies dealing with similar heterocyclic compounds, as indicated by Eissa *et al.* [34]. Yet the demonstrated moderate activity of these two compounds against MDA-MB-213 relative to doxorubicin suggests that their mechanism of activity is different and their target cell survival pathways are potentially different given that the MDA-MB-213 is a triple-negative breast cancer model.

The amino pyridine moiety may impart specific interaction with molecular targets unique to this aggressive breast cancer subtype. This is similar to the findings of Gurdal *et al.* [35], who found that piperazine derivatives containing a nitrogen heterocycle exhibited selective cytotoxicity, possibly *via* interactions that regulate microtubule dynamics or the kinase pathway. The IC_{50} of 40 ± 2.5 μM for **3i**, while higher than that of **3g** and **3h** suggests a moderate potency and opportunities for structural optimisation to develop more effective compounds. In comparison, the literature shows efficacy of other heterocyclic compounds against similar cell lines. For example, Rao *et al.* [33] reported benzamide-piperazine-sulphonamide hybrids with IC_{50} of 24.2-38.2 μM against MDA-MB-231 and HeLa cell lines, which indicate that com-pounds **3g** and **3h**, with lower IC_{50} have moderate potency.

Conclusion

A novel series of piperazine derivatives incorporating the 1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)cyclopropane-1-carboxylic acid scaffold was successfully synthesised, characterised and evaluated for cytotoxic activity against MCF-7, MDA-MB-231 and HeLa cell lines. Compounds **3g** and **3h** exhibited pronounced cytotoxic activity across all three cell lines, whereas compound **3i** displayed selective activity toward MDA-MB-231 cells. Molecular docking of compounds **3g**, **3h** and **3i** with ERα and EGFR revealed favourable binding interactions, with compound **3h** displaying the highest binding affinity among the synthesised derivatives. The docking results were consistent with the *in vitro* cytotoxicity and provided structural insight into the observed biological activity. Thus, based on these findings identify compounds **3g**, **3h** and **3i** as valuable lead structures for further structural optimisation and biological evaluation in the search for more potent anticancer agents.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language and no data, results or scientific conclusions were generated by AI. All analyses, interpretations and conclusions are the authors' own. The authors reviewed and edited the content and take full responsibility for the published work.

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