Synthesis, biological evaluation and molecular modeling of novel benzofuran-*N*- heterocyclic hybrids as anticancer agents

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Abstract: A novel series of benzofuran scaffolds hybridized with 2-substituted thiazoles **6a-c** and **7a-c**, pyrazoles **9** and **10** and 2-substituted quinolines **11** and **12** was designed and synthesized as potential anticancer agents. The in vitro antitumor activity of these hybrids was evaluated by MTT assay using doxorubicin as positive control against five human cancer cell lines including hepatocellular carcinoma (HePG2), colorectal carcinoma (HCT-116), breast carcinoma (MCF-7), human prostate carcinoma (PC3) and cervical carcinoma (Hela). Benzofuran-pyrazole hybrids 9 and 10 and benzofuran-thiazole 6a were identified as the most promising anticancer candidates against HCT-116 and PC3. Further investigation revealed that compounds 6a exhibited high inhibitory activity against Topoisomerase 1 and induced apoptosis at Pre-G phase and cell cycle arrest at G2/M phase in HCT-116 cells.
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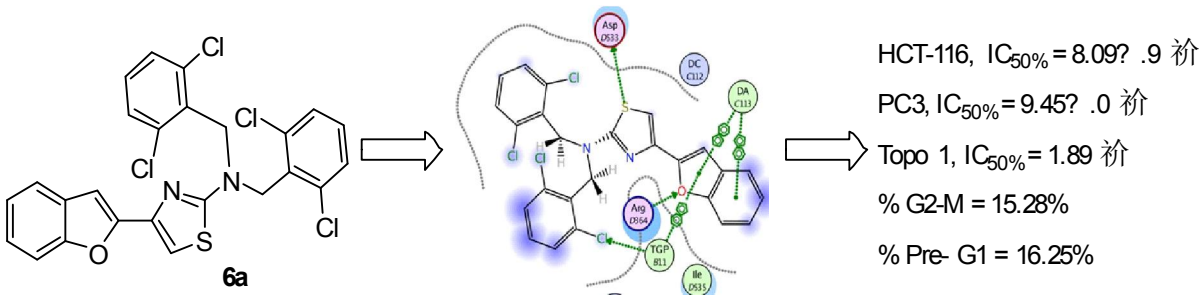
Keywords: Benzofuran, hybrids, thiazole, pyrazole, quinoline, anticancer agents, Topoisomerase, apoptosis and cell cycle arrest.

Graphical Abstract



1. Introduction

Cancer is a very complicated disease; it affects different organs and systems of the body. It is now the most common cause of mortal reasons worldwide, which is scourge on humanity. Therefore, there is an urgent need to synthesize new potent and selective chemotherapeutic drugs to fight cancer and continue human's battling against cancer (Chen, Chen et al. 2009, Omaima M. Abdelhafez, Eman Y. Ahmed et al. 2014).

The vast majority of literatures indicated that heterocyclic compounds have attracted considerable interest due to their valuable biological activities especially antitumor activity. They are essential parts of many clinically useful chemotherapeutic agents(Martins, Jesus et al. 2015). Among of these heterocycles, natural benzofurans-containing products such as ebenfuran III and moracins O were identified to possess potent antitumor activity as shown in Figure1(Yang, Wan et al. 2012, Wang, Liu et al. 2013).

The heterocyclic moieties like aminothiazole, pyrazole and quinoline proved to have interesting structures that exhibit versatile biological properties including anti-inflammatory, antimicrobial, antifungal, antioxidant and antitumor activity. As shown in Figure 1, compounds **1**, **2** and topotecan were proved to have potent antitumor activity against hepatocellular carcinoma, breast carcinoma and lung carcinoma(Chimenti, Bizzarri et al. 2007, Potewar, Ingale et al. 2008, Bandgar, Gawande et al. 2009, Suresh Kumar 2009, Guzeldemirci and Kucukbasmaci

2010, Choi, Lee et al. 2012, Afzal, Kumar et al. 2015, Eldehna, Altoukhy et al. 2015, Somaia S. Abd El-Karim, Manal M. Anwar et al. 2015).

A hybrid pharmacophore approach is a technique which combine two or more pharmacophores into single molecule, which could be beneficial for developing new chemotherapeutic agents with different modes of action, decreasing unwanted side effects and overcoming drug resistance (Solomon, Hu et al. 2010, Rida, Youssef et al. 2012).

Based on these findings, this work aims to design new hybrids structurally containing benzofuran and *N*-heterocyclic ring system and to emulate them for their antitumor activity against five selected tumor cell lines.

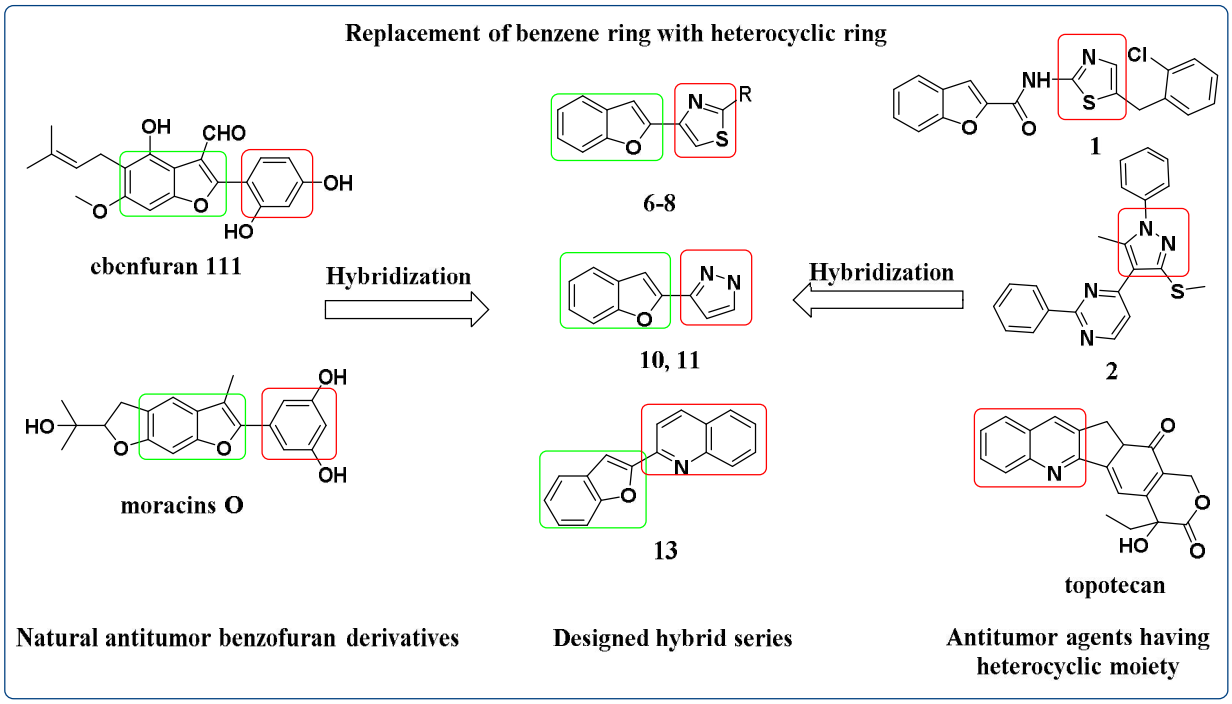


Figure 1. Design of novel benzofuran-*N*-heterocyclic hybrids as anticancer agents.

2. Chemistry

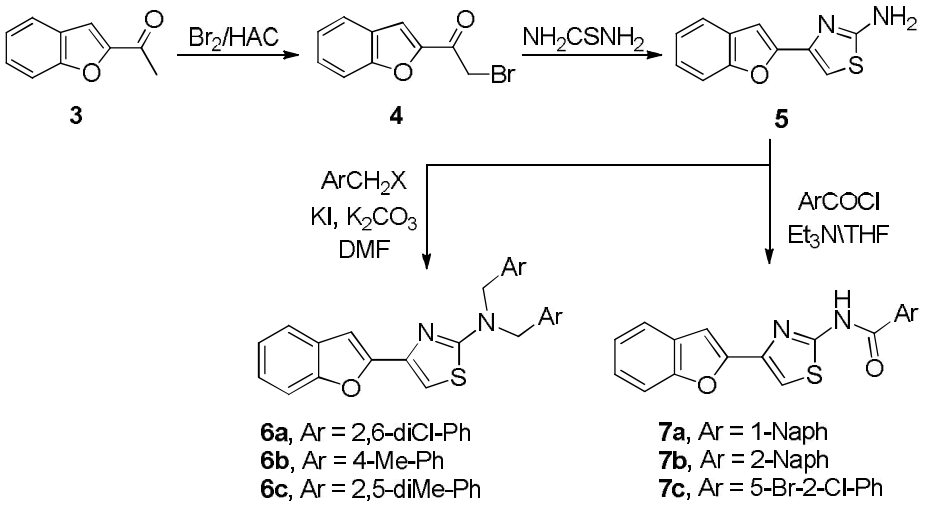
Our target series of the novel benzofuran-thiazole hybrids were prepared as shown in Scheme 1. Bromination of 2-acetyl benzofuran (**3**) in acetic acid afforded bromo derivative **4** according to the reported procedure (Kumar, Prakash et al. 2006, Metwally, Abdel-Wahab et al. 2010). 2-Aminothiazole-benzofuran (**5**) was prepared in a good yield through Hantzsch thiazol synthesis by treating thiourea with 2-bromo acetylbenzofuran (**4**) in ethanol under reflux (Venkatesh, Bodke et al. 2010, Annadurai, Martinez et al. 2012). Compound **5** is reacted with benzyl halide derivatives in the prescence of K_2CO_3 and KI in DMF to give the *N*, *N*-bisubstituatedbenzyl amino thaizoles **6a-c**. Also, compound **5** is reacted with acid chloride derivatives in presence of triethylamine in THF to produce benzofuran-thiazole-substituted amide **7a-c**. Comparative data for novel hybrids with respective to structures and yield are provided in Table 1.

As shown in Scheme 2, the dicyanointermediate **8** was obtained through Knovengeal condensation of 2-acetyl benzofuran (**3**) with malononitrile in ethanol according to the reported procedure (Villemin, Belhadj et al. 2013) and the structure of compound **8** was confirmed by the appearance of the absorption band at 2210 cm^{-1} for (CN) in the IR spectrum. Moreover, the ring closure of compound **8** with hydrazine hydrate in ethanolic solution led to the formation of 4-(1-benzofuran-2-yl) ethylidene)-4*H*-pyrazole-3,5-diamine (**9**) according to the reported procedure (Selim 1998)which confirmed by absorption band at 3377 $cm^{-1}$for (NH_2), While the reaction with phenyl hydrazine gave 5-amino-3-(benzylfuran-2-yl)-3-methyl-2-phenyl-2,3dih -ydro-1*H*-pyrazole-4-carbonitrile (**10**) (M'Hamed and Alduaij 2016)which confirmed by absorption bands of three functional group of (NH_2), (NH) and (CN) at 3422, 3309 and 2215, respectively.

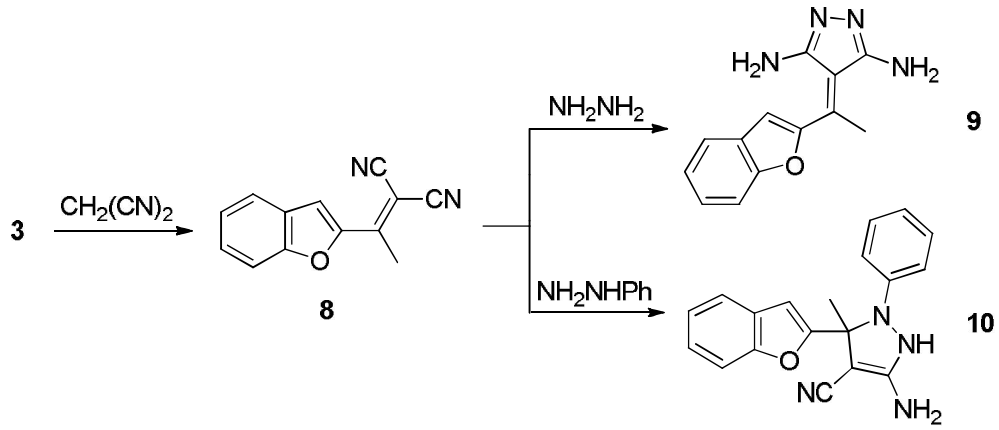
A possible mechanism for the formation the diamine pyrazole (**9**) was described in Figure 2. One of the nitrile groups was attacked by a molecule of hydrazine hydrate to give intermediate **A**, which followed by intramolecular cyclization between the amino group and the other nitrile group to give the

cyclic intermediate **B**. Aromatization is the last step to form pyrazole-diamine nucleus.

New benzofuryl quinoline hybrids **11** and **12** were prepared as shown in Scheme 3. Hybrid **11** was formed through the reaction of 2-aceyl benzofuran (**3**) with 5-flouroistatin *via* Pfitzinger reaction in alcoholic KOH (Shankerrao, Bodke et al. 2012, Bodke, Shankerrao et al. 2013). The condensation of thiosemicarbazide with carboxylic acid **11** produced the thiadiazole derivative **12** (Ramjith U. S, Radhika G et al. 2013). The structure of **11** was confirmed by the presence the carboxylic proton at 14.2 ppm and the disappearance of the methyl protons of compound **3** as indicated in the ^{1}H-NMR spectra. Comparative data for novel hybrid compounds and yield are provided in Table 1.



Scheme 1. Synthesis of benzofuran-thiazole hybrids **6** and **7**.



Scheme 2. Synthetic route of benzofuran-pyrazole hybrids **9** and **10**.

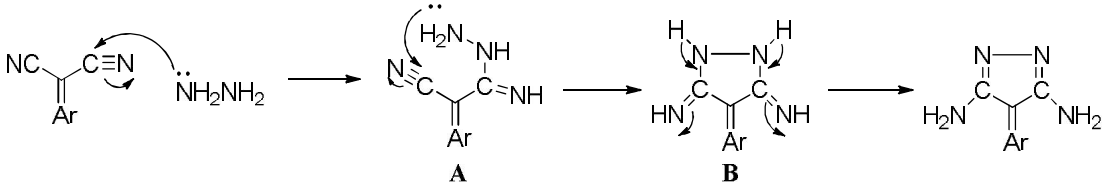
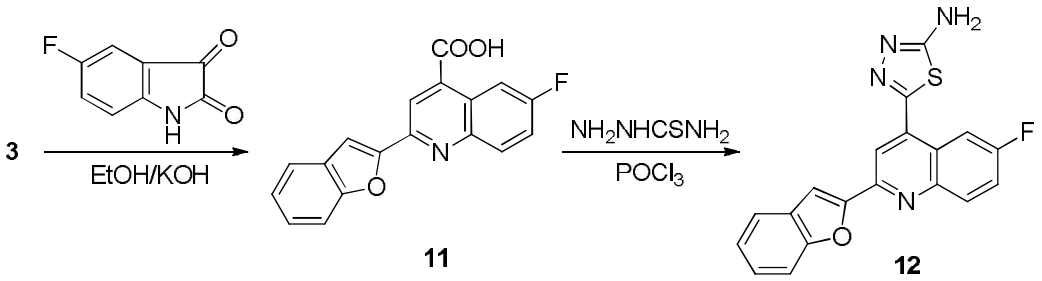


Figure 2. Mechanism of the formation of hybrid **9**, Ar = benzofuryl ring.



Scheme 3. Synthetic route of benzofuran-quinoline hybrids **11** and **12**.

Table 1. Comparative data of novel hybrids **6-12**

Compound	Molecular Formula	MP (°C)	Yields (%)
6a	$C_{25}H_{16}C_{l4}N_2OS$	110-115	55
6b	$C_{27}H_{24}N_2OS$	166-170	56
6c	$C_{29}H_{28}N_2OS$	156-160	53
7a	$C_{22}H_{14}N_2O_2S$	139-144	50
7b	$C_{22}H_{14}N_2O_2S$	>300	45
7c	$C_{18}H_{10}BrClN_2O_2S$	154-159	58
8	$C_{13}H_8N_2O$	140-144	90
9	$C_{13}H_{12}N_4O$	148-152	61
10	$C_{19}H_{16}N_4O$	88-93	66
11	$C_{18}H_{10}FNO_3$	173-178	70
12	$C_{19}H_{11}FN_4OS$	>300	98

3. Biology

3.1 Cytotoxicity screening

The cytotoxic activities of the novel synthesized hybrids **6-12** were evaluated against human cancer cell lines using doxorubicin (DOX) as a reference drug. The cancer cells include hepatocellular carcinoma (HePG2), colorectal carcinoma (HCT-116), breast carcinoma (MCF-7), human prostate carcinoma (PC3) and cervical carcinoma (Hela). The biological results of the tested compounds are summarized in Table 2 ($IC_{50\%}$ values, defined as the concentration of compound that inhibits 50% of the cell growth.

As shown in Table 2, the structures of hybrid compounds have an obvious influence on anticancer activities. Among the disubstituted amino thiazole derivatives **6a-c**, compound **6a** showed the highest antitumor activity against the five cell lines with IC_{50} values ranging from 8.09±0.9 μM to 14.04±1.2 μM and the most sensitive cell line was HCT-116. While the amide derivatives **7a-c** showed a lower activity than **6a** with IC_{50} ranging from12.94±1.2 μM to 47.38±2.9 μM. Amide **7a** strongly inhibits the growth of HCT-116 and PC3 with IC_{50} values 13.44±1.2 μM and 12.94±1.2 μM, respectively.

Table 2. *In vitro* cytotoxic activities (IC_{50}, μM)[a,b] of the hybrids **6-12**.

Compound	HePG2	HCT-116	MCF-7	PC3	Hela
6a	**14.04±1.2**	**8.09±0.9**	**11.62±1.1**	**9.45±1.0**	**10.23±1.1**
6b	71.02±3.9	54.71±3.0	62.94±3.5	56.82±3.1	59.23±3.3
6c	75.39±4.3	68.31±3.8	85.96±4.2	78.01±3.9	89.16±4.4
7a	32.86±2.3	13.44±1.2	41.64±2.6	12.94±1.2	25.20±1.7
7b	30.68±2.1	15.33±1.1	39.44±2.2	16.39±1.4	27.32±1.5
7c	21.41±1.6	47.38±2.9	29.73±1.9	26.10±1.7	33.21±2.0
9	15.47±1.2	11.88±1.1	10.27±1.0	24.15±1.9	13.84±1.2
10	**5.60±0.7**	**9.51±0.9**	**8.18±0.7**	**15.00±1.4**	**8.44±0.8**
11	38.53±2.3	63.23±3.6	47.02±2.5	64.13±3.5	58.23±3.2
12	23.06±1.5	43.15±2.4	28.13±1.8	36.15±2.7	30.41±2.3
DOX	4.50±0.2	5.23±0.3	4.17±0.2	8.87±0.6	5.57±0.4

[a]IC_{50} values for each cell line is the concentration of compound that inhibits 50% of the cell growth measured by MTT assay. [b]Each value was reproduced in triplicate.

Surprisingly, the resulting pyrazole hybrids **9** and **10** show strong and broad antitumor activity against the five cell lines with IC_{50} values ranging from 10.27±1.0 µM to 24.15±1.9 µM for compound **9** and 5.60±0.7 µM to 15.00±1.4 µM for compound **10**. Moreover, quinoline-containing derivatives **11** and **12** exhibited moderate inhibition activity and **12** showed the best activity towards HePG2 cell line with IC_{50} equal to 23.06±1.5 µM.

The results indicated that hybridization between benzofuran and different *N*-heterocyclic moieties exhibited remarkable growth inhibitory effect against the tested cancer cells. The most potent hybrids are pyrazole-containing derivatives especially compound **10**.

3.2 Enzyme inhibition assay

Table 3. Inhibitory activities (IC_{50} µM) against Top1 and aromatase enzymes.

Compound	IC_{50} (µM)	Top1 IC_{50} (µM)	Aromatase IC_{50} (µM)
6a	HCT-116 (8.09±0.9)	1.89	-
11	MCF-7 (8.18±0.7)	-	4.40
	HePG2(5.60±0.7)	-	16.41
Camptothecin	HCT-116	1.1	-
letrazole	MCF-7	-	0.28
	HePG2	-	0.24

In order to evaulate the mode of action of the synthesized compounds as anticancer agents. Compound **6a** was tested for its topoisomerase 1 (Top 1) inhibition activity. In addition, compound **10** was tested for its aromatase inhibition activity.

As shown in Table 3, hybrid **6a** show inhibitory activity in HCT-116 cells (IC_{50} = 2.15 µM) against.

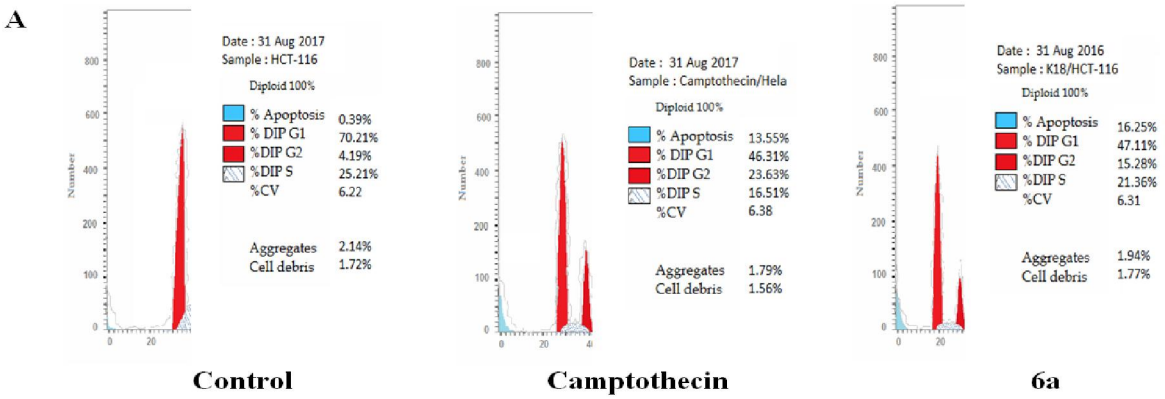
Top 1 and closer activity to Camptothecin (IC_{50} = 1.1 µM) as a reference drug. This result indicates that Top 1 may be a possible target for the designed hybrids for their antitumor activity. In addition, hybrid **10** exhibited a weak inhibitory activity towrads aromatase enzyme in MCF-7 and HePG2 cancer cells (IC_{50} = 4.4 and 16.41 µM, respectively) in comparison to letrazole as aromatase inhibitor.

3.3 Cell-cycle analysis

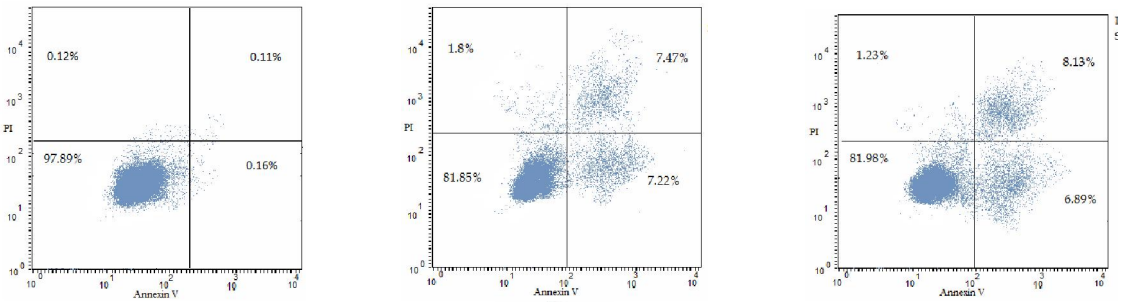
The cytotoxic effect of the anticancer drugs could be the result of cell cycle arrest, or induction of apoptosis (programmed cell death), or a dual effect of both modes. Therefore, the effect of compound **6a** on cell cycle distribution was screened in HCT-116 cells. HCT-116 cells were treated with 6.351 µM of **6a** for 24 h and then stained with propidium iodide. DNA contents were measured by flow cytometry (Figure 3A). Compared with control which was treated with DMSO, the cell proportion at S phase after treating HCT-116 cells with **6a** decreased to 21.36 %. In addition, **6a** increased the cell proportion at G2/M phase to 15.28 % in comparison to the control cells (4.19 %), which means that the cells were arrested at G2/M phase. On the other hand, there was pre-G1 population detected after treatment with **6a** which was 16.25 %, when compared to the controlled cells (0.39 %), suggesting that hybrid **6a** might induce apoptosis in HCT-116 cells. As shown in Figure 3B, an increase of apoptotic cell population was detected in the **6a** treated cells for both 24 h compared with that in HCT-116 cells without treatment. This result indicates that compound **6a** isapoptosis inducer, which is consistent with the data obtained from cell cycle analysis in Figure 3A.

Table 4. Effect of compound **6a** on HCT-116 cell cycle progression using flow cytometry.

Compound	Cell cycle distribution (%)			
	G0/G1	S	G2/M	Pre/G1
Control	70.21	25.21	4.19	0.39
Camptothecin	48.37	13.34	21.71	16.49
6a	47.11	21.36	15.28	16.25



B



Control **Camptothecin** **6a**

Figure 3. **A)** Effect of compound **6a** on the cell cycle distribution of HCT-116 cell line. **B)** Apoptosis effect on human HCT-116 cell line induced by compound **6a**.

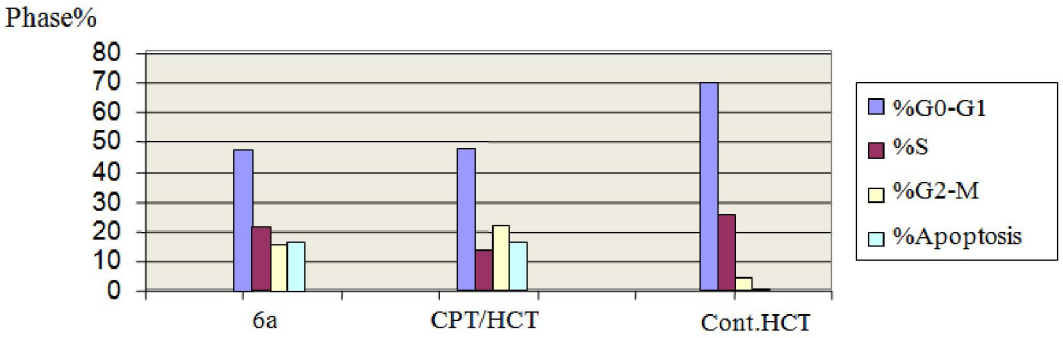


Figure 4. Cell cycle analysis for hybrid **6a** in comparsion with camptothecin and control cell (HCT-116).

Table 5. Apoptotic activity of compound **6a**.

Compound	Total	Early apoptosis	Late apoptosis	Necrosis
Control/HCT-116	0.39	0.16	0.11	0.12
Camptothecin/HCT-116	16.49	7.22	7.47	1.8
6a	16.25	6.89	8.13	1.23

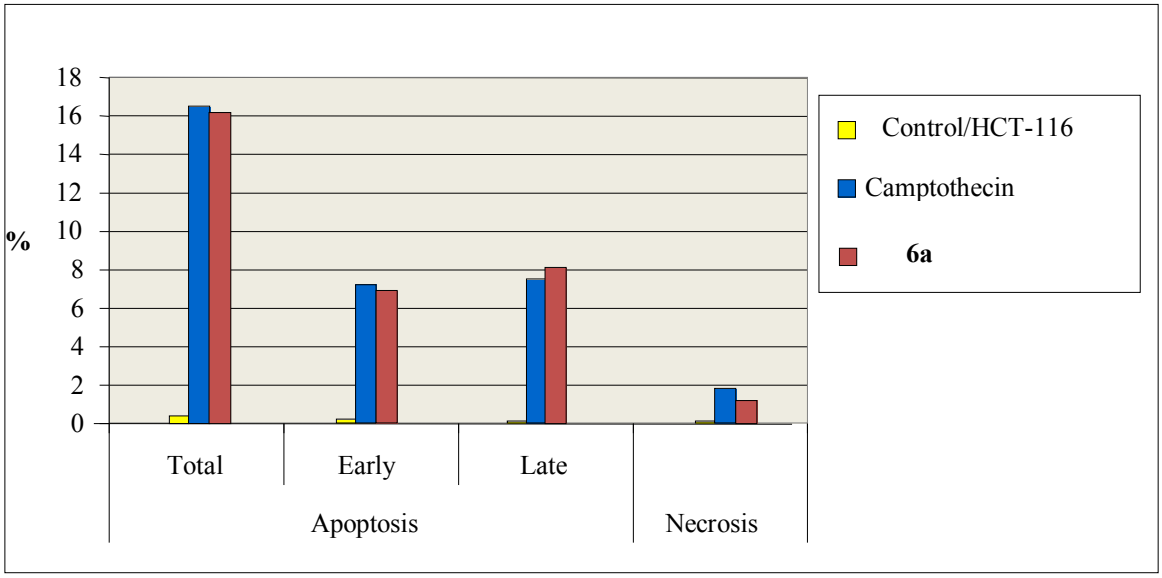


Figure 5. Apoptosis for hybrid **6a** in comparsion with camptothecin and control cell (HCT-116).

4. Docking study

Topoisomerase 1 (Top1) is ubiquitous enzyme which relax supercoiled DNA during various cellular events viz. replication, transcription and recombination. In various tumor cells, Top1 is expressed much higher than the normal cells, hence, modulating the Top1 levels could be an essential therapeutic treatment for cancer [27, 28].

Molecular modeling calculations and docking studies were performed using molecular operating environment (MOE) software version 2014.09 (Chemical Computing Group Inc., Montreal, Quebec, Canada) with the aim of explaining the promising Top1 inhibitory activity of the newly synthesized compounds through investigating their binding mode and their interaction with the key amino acids (hot spots) in the active site of the Top1.

Molecular docking studies of hybrid **6a** was carried out using the crystal structure of HTop1 viz. receptor (PDB ID: 1K4T) a Top1 in complex with poison topotecan (camptothecin derivative) which obtained from protein data bank. The key amino acids in the active side of the Top1 are **Arg364**, **Asp533**, **Asn722** (Bart L. Staker, Michael D. Feese et al. 2005). Figure 6explains the binding activity of topotecan in the active site of Top1.

When examining the protein-ligand interaction for the protein molecules (**1K4T**) with hybrids. Compound **6a** formed two H-bonds (hydrogen bonds) with active site residues **Arg364**, **Asp533** which are known to be main amino acids involved in the binding and required for Top1 sensitivity to camptothecin (Bart L. Staker, Michael D. Feese et al. 2005). One of the H-bonds occurred between O-benzofuran of compound **6a** and NH_2 of **Arg 364** and the second between S-aminothiazole of compound **6a** and OD2 of **Asp533**, Also hybrid **6a** formed π-π stacking interaction between benzofuran ring and deoxy adenine.

Considering the molecular docking studies (Figure7), they are consistent with our experimental data showing that compound **6a** stabilizes Human Top1-DNA cleavage complex like CPT and inhibits Top1 activity.

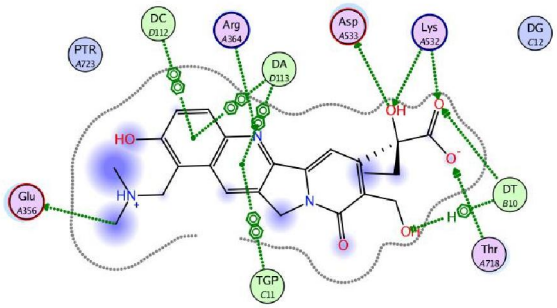


Figure 6. 2D diagram of topotecan (CPT derivtives) showing its interaction with the Top 1 receptor active site.

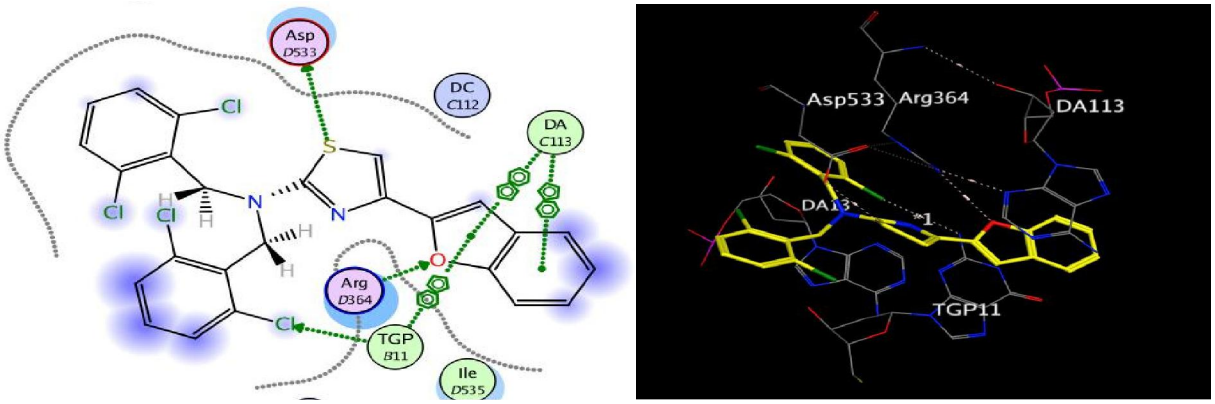


Figure 7. 2D and 3D diagram of hybrid **6a** showing its interaction with the Top 1 receptor active sit

Conclusion

Several hybrids of benzofuran-*N*-heterocyclic moieties were synthesized and evaluated against five human cancer cell lines for their antitumor activities. Benzofuran-containing thiazole **6a** and pyrazole **10** hybrids were identified as the most promising anticancer candidates against HCT-116 and PC3. The inhibitory activity against Topoisomerase 1 and induction of apoptosis of **6a** were further investigated in HCT-116 cells. The result revealed that hybrid **6a** has Top 1 inhibitory activity similar to camptothecin and it is apoptosis inducers. These biochemical data, consistent with the molecular docking study for hybrid **6a**.

6.1. Chemistry

Melting points were recorded using Fischer-John apparatus and are uncorrected. IR spectra were recorded on Nicolet IS 10 FT-IRspectrometer in KBr

discs. The ^{1}H-NMR and ^{13}C-NMR were determined by using Burker 400 MHz or Joel 500 MHz. All chemical shift values, coupling constants *J* and the multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad) are quoted in ppm and in Hz, respectively using TMS as internal standard. The mass spectra were detected using Hewlett Packard 5988 spectrometer. TLC monitored the reactions on silica gel precoated aliuminium sheets and the spots were detected by UV lamp at wave length 254 nanometer. Compounds 1-(Benzofuran-2-yl)-2-bromoethan-1-one (**4**) and 1-(Benzofuran-2-yl)-2-bromoethan-1-one (**5**) were prepared according to the described method(Kumar, Prakash et al. 2006, Metwally, Abdel-Wahab et al. 2010),(Venkatesh, Bodke et al. 2010, Annadurai, Martinez et al. 2012). All chemicals and reagents were purchased from Aldrich, Fluca or Merck and used without further purification.

6.1.1. Synthesis of 1-(benzofuran-2-yl)-2-bromoethan-1-one (**4**)(Kumar, Prakash et al. 2006, Metwally, Abdel-Wahab et al. 2010)

To a solution of 2-acetyl benzofuran (**3**) (2.0 g, 12.5 mmol) in acetic acid (15 ml), a solution of bromine (0.875 g, 12.5 mmol, 2.0 ml) in acetic acid (10 ml) was slowly added and stirred at room temperature for 24 h. The mixture was poured over ice water during vigorous stirring. The separated solids were filtered, washed with water and dried to give the titled compound as yellow solids m.p. = 87-90 °C (1.55 g, yield 77.5%). Reported m.p. = 89-90 °C(Jakhar and Makrandi 2008)

6.1.2.Synthesis of 4-(benzofuran-2-yl)thiazol-2-amine (**5**)(Venkatesh, Bodke et al. 2010, Annadurai, Martinez et al. 2012)

A mixture of compound **4** (2.0 g, 8.36 mmol) and thiourea (0.63 g, 8.36 mmol) in ethanol (15ml) was refluxed for 3 h. The reaction mixture was cooled to rt and neutralized with dilute ammonia till pH = 12. The separated solids were filtered and crystallized from ethanol and dried to give the titled compound as yellow solids with m.p. = 212-215°C (1.78g, yield = 99%). Reported m.p. = 214 °C(Kumar, Prakash et al. 2006).

6.1.3. General procedure for the preparation 4-(benzofuran-2-yl)-*N*-(substituted benzyl) thiazol-2-amine6a-c

A mixture of compound **5** (0.324 g, 1.5 mmol) and substituted benzyl halides (1.5 mmol), K_2CO_3 (0.414 g, 3.0 mmol) and KI (0.049 g,0.3 mmol) in DMF (8 ml) was stirred at rt for 24 h. The reaction mixture was diluted with EtOAc (10 ml) and followed by addition of brine (5 ml). The aqueous layer was extracted with EtOAc (10 ml x 3) and the organic extracts were dried over anhydrous Na_2SO_4, filtered, washed and evaporated under reduced pressure. The obtained residue was purified by column chromatography (SiO_2, petroleum ether/ EtOAc = 5:1) to give the titled products **6a-c**.

6.1.3.1. Synthesis of 4-(benzofuran-2-yl)-*N*, *N*-bis (2,6-dichlorobenzyl) thiazol-2-amine (**6a**)

Yellow solid with m.p. = 110-115 °C, Yield = 55%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.57 (s, 2H, CH_2), 5.72 (s, 2H, CH_2), 7.03 (dd, *J* = 15.8, 8.0 Hz, 1H, ArH), 7.11 (d, *J* = 4.8 Hz, 2H, ArH), 7.13 (s, 1H, ArH), 7.23 (d, *J* = 7.0 Hz, 1H, ArH), 7.30 (d, *J* = 6.4 Hz, 2H, ArH), 7.34 (d, *J* = 5.6 Hz, 1H, ArH), 7.37 (d, *J* = 3.8 Hz, 1H, ArH). IR (KBr, cm^{-1}): 3449, 2964, 1707, 1503, 1394, 1228, 1013, 808, 737, 651. ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 45.97, 63.14, 102.44, 110.71, 111.77, 121.13, 123.00, 124.48, 127.98, 128.31, 129.38, 129.47, 131.28, 131.53, 134.47, 135.92, 139.55, 151.09, 152.84, 153.79, 160.45. Elemental Analysis for $C_{25}H_{16}C_{l4}N_2OS$, Calcd: C, 56.20; H, 3.02; N, 5.24; Found, C, 56.21; H, 3.03; N, 5.25.

6.1.3.2. Synthesis of 4-(benzofuran-2-yl)-*N*, *N*-bis (4-methylbenzyl) thiazol-2-amine (**6b**)

Yellow solid with m.p. = 166-170 °C, Yield = 56%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.34 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 5.31 (s, 2H, CH_2), 5.42 (s, 2H, CH_2), 7.10 (s, 1H, ArH), 7.12 (s, 2H, ArH), 7.21 (d, *J* = 8.0 Hz, 2H, ArH), 7.26 (d, *J* = 7.6 Hz, 2H, ArH), 7.30 (d, *J* = 10.0 Hz, 2H, ArH), 7.34 (d, *J* = 7.6 Hz, 2H, ArH), 7.38 (s, 1H, ArH), 7.52 (d, *J* = 8.0 Hz, 1H, ArH), 7.62 (d, *J* = 7.2 Hz, 1H, ArH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 20.73, 49.46, 68.50, 102.91, 111.06, 111.95, 121.50, 123.33, 124.80, 127.64, 128.32, 128.99, 132.26, 134.07, 136.61, 137.77, 140.13, 151.56, 154.18, 161.19. IR (KBr, cm^{-1}): 3449, 1503, 1394, 1228, 737. Elemental Analysis for $C_{27}H_{24}N_2OS$, Calcd: C, 76.38; H, 5.70; N, 6.60; Found: C, 76.37; H, 5.72; N, 6.61.

6.1.3.3. Synthesis of 4-(benzofuran-2-yl)-*N*, *N*-bis (2,5-dimethylbenzyl) thiazol-2-amine (**6c**)

Yellow solid with m.p. = 156-160 °C, Yield = 53%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.15 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.30 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 5.30 (s, 2H, CH_2), 5.45 (s, 2H, CH_2), 6.82 (s, 1H, ArH), 6.96 (d, *J* = 7.6 Hz, 1H, ArH), 7.01 (s, 2H, ArH), 7.04 (s, 1H, ArH), 7.06 (s, 2H, ArH), 7.23 (t, 7.3 Hz, 1H, ArH), 7.29, 2H, ArH), 7.40 (s, 1H, ArH), 7.50 (d, *J* = 8.1 Hz, 1H, ArH), 7.57 (d, *J* = 7.5 Hz, 1H, ArH). IR (KBr, cm^{-1}): 3450, 2922, 1501, 1244, 1140, 1020, 955, 740. Elemental Analysis for $C_{29}H_{28}N_2OS$, Calcd: C, 76.96; H, 6.24; N, 6.19; Found: C, 76.95; H, 6.25; N, 6.17.

6.1.4. General procedure for the preparation *N*-(4-(benzofuran-2-yl) thiazol-2-yl)-2-substituated amide 7a-c

To a mixture of compound **5** (1.0 equiv) and TEA (2.0 equiv) in THF (3 ml), a solution of an

appropriate acid chloride in THF (5 ml) was added at 0 °C, the acid chloride was prepared from the corresponding aromatic acid (1.5 mmol) through refluxing with $SOCl_2$ (5 ml). The reaction mixture was warmed to 25 °C and stirred for 24 h. The reaction mixture was concentrated under reduced pressure and the residue was diluted with EtOAc (10 ml) and brine (5 ml). The aqueous layer was extracted with EtOAc (10 ml x 3) and the organic extracts were collected and dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The obtained residue was purified by column chromatography (SiO_2, petroleum ether/ EtOAc = 5:1) to give the titled products **7a-c**.

6.1.4.1. Synthesis of *N*-(4-(benzofuran-2-yl) thiazol-2-yl)-1-naphthamide (7a)

Yellow solid with m.p. = 139-144 °C, Yield = 50%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.18 (s, 1H, ArH), 7.54 (m, 2H, ArH), 7.64 (dd, *J* = 8.0, 7.6 Hz, 2H, ArH), 7.88 (d, *J* = 8.0 Hz, 2H, ArH), 8.08 (d, *J* = 8.0 Hz, 2H, ArH), 8.37 (d, *J* = 7.2 Hz, 2H, ArH), 9.08 (d, *J* = 8.8 Hz, 2H, ArH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 124.90, 125.48, 126.21, 127.62, 128.62, 129.89, 130.67, 132.97, 133.47, 186.64. IR (KBr, cm^{-1}): 3450 (NH), 3052, 1770 (C=O), 1510, 1224, 1170, 1058, 961, 770. MS (EI) *m/z* ($C_{22}H_{14}N_2O_2S$): 370 (M^+, 1.59%), 326.10 (26.88%), 155.03 (81.46%), 127.07 (100%), 101.06 (6.0%), 51.04 (1.34%).Elemental Analysis for $C_{22}H_{14}N_2O_2S$, Calcd: C, 71.33; H, 3.81; N, 7.56; Found: C, 71.31; H, 3.82; N, 7.55.

6.1.4.2 Synthesis of *N*-(4-(benzofuran-2-yl) thiazol-2-yl)-2-naphthamide (7b)

Yellow solid with m.p. = over 300 °C, Yield = 45%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.08 (s, 1H, ArH), 7.45 (s, 1H, ArH), 7.55 (dd, *J* = 5.9, 3.3 Hz, 1H, ArH), 7.62 (d, *J* = 7.5 Hz,, 1H, ArH), 7.67 (d, *J* = 6.7 Hz, 1H, ArH), 7.72 (dd, *J* = 5.6, 3.3 Hz, 1H, ArH), 7.90 (d, *J* = 8.4 Hz, 2H, ArH), 7.97 (m, 2H, ArH), 8.07 (t, *J* = 9.6 Hz, 1H, ArH), 8.61 (d, *J* = 19.4 Hz, 1H, ArH). Elemental Analysis for $C_{22}H_{14}N_2O_2S$, Calcd: C, 71.33; H, 3.81; N, 7.56; Found: C, 71.32; H, 3.80; N, 7.54.

6.1.4.3 Synthesis of *N*-(benzofuran-2-yl) thiazol-2-yl)-5-bromo-2-chlorobenzamide (7c)

Yellow solid with m.p. = 154-159 °C, Yield = 58%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.83 (s, 1H, ArH), 6.97 (d, *J* = 8.6 Hz, 1H, ArH), 7.21 (dd, *J* = 6.9, 6.9 Hz, 1H, ArH), 7.28 (dd, *J* = 9.0, 9.0 Hz, 2H, ArH), 7.40 (d, *J* = 5.0 Hz, 1H, ArH), 7.45 (d, *J* = 8.1 Hz, 1H, ArH), 7.53 (d, *J* = 7.7 Hz, 1H, ArH), 7.67 (dd, *J* = 3.1 Hz, 1H, ArH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 112.32, 114.50, 118.12, 123.80, 124.20, 126.78, 128.68, 134.92, 151.55, 154.93, 183.36, 194.03. IR (KBr, cm^{-1}): 3163, 2923, 1678 (C=O), 1555, 1442, 1302, 814, 744, 504. MS (EI) *m/z* ($C_{18}H_{10}BrClN_2O_2S$): 436.95 (M+2, 6.85%), 433.96 (M^+, 100%), 396.95 (53.08%), 218.92 (73.32%), 190.92 (19.18%), 75.06 (6.97%). Elemental Analysis for $C_{18}H_{10}BrClN_2O_2S$, Calcd: C, 49.85; H, 2.32; N, 6.46; Found: C, 49.84; H, 2.31; N, 6.45.

6.1.5. Synthesis of 2-(1-benzofuran-2-yl) ethylidene) malononitrile (8)

To a solution of 2-acetyl benzofuran (**3**) (0.32 g, 2.0 mmol) in EtOH (15 ml) was added malononitrile (0.132 g, 2.0 mmol) and the mixture was refluxed overnight. After cooling and evaporating ethanol under reduced pressure yellow crystals were formed and collected by filtration and recrystallized from EtOH to give the titled compound as yellow needle crystals with m.p. = 140-144 °C (0.37 g, yield = 90%).^{1}H-NMR (500 MHz, DMSO-d_6): δ = 2.63 (s, 3H, CH_3), 7.42 (dd, *J* = 8.9, 8.9 Hz, 1H, ArH), 7.60 (dd, *J* = 9.3, 9.3 Hz, 1H, ArH), 7.70 (d, *J* = 10.5 Hz, 1H, ArH), 7.87 (d, *J* = 9.6 Hz, 1H, ArH), 8.08 (s, 1H, ArH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 19.38, 78.23, 111.56, 113.24, 113.60, 117.39, 123.23, 124.28, 126.83, 129.83, 149.91, 155.11, 156.94. IR (KBr, cm^{-1}): 3743, 3618, 2222 (CN), 1697, 1610, 1572, 1530, 1429, 1315, 1189, 1142, 943, 824, 750, 612, 547. MS (EI) *m/z* ($C_{13}H_8N_2O$): 209.08 (M+1, 6.05%), 208 (M^+, 39.53%), 199.05 (20.28%), 167.08 (13.06%), 153.08 (7.61%), 143.04 (4.71%), 118.06 (6.95%), 94.07 (15.20%), 67.08 (6.83%), 44.03 (25.09%). Elemental Analysis for$C_{13}H_8N_2O$, Calcd: C, 74.99; H, 3.87; N, 13.45; Found: C, 74.98; H, 3.88; N, 13.46.

6.1.6. General procedure for the preparation compounds 9 and 10

A mixture of compound **8** (0.2 g, 0.96 mmol) and hydrazine derivative (0.96 mmol) in ethanol (10 ml) was refluxed and stirred overnight. The reaction mixture was cooled to rt and the formed precipitate was filtered and recrystallized from EtOH to give the titled compounds as yellow solids with m.p. = 148-152 °C (0.14 g, yield = 61%) for **9** and red solids with m.p. = 88-93 °C (0.2 g, yield = 66%) for **10**.

6.1.6.1. Synthesis of 4-(1-benzofuran-2-yl) ethylidene)-4*H*-pyrazole-3,5-diamine (9)

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 2.03 (s, 3H, CH_3), 7.21 (s, 2H, $2NH_2$), 7.22 (s, 1H, ArH), 7.23 (d, *J* = 10 Hz, 2H, ArH), 7.25 (d, *J* = 9.5 Hz, 1H, ArH), 7.26 (s, 1H, ArH). IR (KBr, cm^{-1}): 3377(NH_2), 3277, 1578, 1141, 1160, 785, 744, 522. Elemental Analysis for $C_{13}H_{12}N_4O$, Calcd: C, 64.99; H, 5.03; N, 23.32; Found: C, 64.98; H, 5.02; N, 23.31.

6.1.6.2. Synthesis of 5-amino-3-(benzylfuran-2-yl)-3-methyl-2-phenyl-2,3dihydro-1*H*-pyrazole-4-carbonitrile (10)

^{1}H-NMR (500 MHz, DMSO-d_6): δ = 2.43 (s, 3H, CH_3), 4.14 (s, 1H, NH), 6.74 (s, 2H, NH_2), 7.08 (s, 1H, ArH), 7.20 (d, *J* = 2.0 Hz, 1H, ArH), 7.30 (dd, *J* =

5.4 Hz, 2.4 Hz, 1H, ArH), 7.42 (m, 1H, ArH), 7.49 (d, *J* = 1.1 Hz, 1H, ArH), 7.52 (s, 1H, ArH), 7.56 (s, 1H, ArH), 7.56 (m, 1H, ArH),7.77 (d, *J* = 7.8 Hz, 1H, ArH), 7.84 (s, 1H, ArH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 12.38, 26.13, 103.36, 110.56, 111.92, 112.68, 113.99, 117.41, 119.08, 120.66, 122.72, 123.35, 123.70, 124.16, 126.53, 128.12, 128.62, 132.28, 145.04, 151.81, 153.91, 154.74,187.58. IR (KBr, cm^{-1}): 3422(NH_2), 3309(NH), 2215(CN), 1601, 1255, 1149, 748. Elemental Analysis for $C_{19}H_{16}N_4O$, Calcd: C, 72.13; H, 5.10; N, 17.71; Found: C, 72.12; H, 5.11; N, 17.73.

6.1.7. Synthesis of 2-(benzofuran-2-yl)-6-fluoroquinoline-4-carboxylic acid (11)

A mixture of compound **3** (0.2 g, 1.32 mmol), 5-flouroisatin (0.48 mmol, 0.08 g) and 33% NaOH solution was refluxed in EtOH (12 ml) and stirred for 24 h. The reaction mixture was cooled to rt and neutralized with acetic acid till pH = 7 and the separated solid was filtrated, washed with water and dried to give the titled compound as red needle crystals with m.p. = 173-178 °C (0.26 g, yield = 70%).^{1}H-NMR (500 MHz, DMSO-d_6): δ = 7.35 (t, *J* = 7.5 Hz, 1H, ArH), 7.46 (dd, *J* = 11.4, 11.4 Hz, H, ArH), 7.78 (dd, *J* = 7.3, 7.3 Hz, 1H, ArH), 7.83 (dd, *J* = 8.4, 8.4 Hz, 1H, ArH), 7.91 (s, 1H, ArH), 8.25 (dd, *J* = 9.3, 9.3 Hz, 1H, ArH), 8.51 (dd, *J* = 11.0, 11 Hz, 1H, ArH), 8.58 (s, 1H, ArH), 14.2 (s, 1H, COOH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 106.94, 109.03, 109.23, 111.40, 119.76, 120.33, 120.53, 121.92, 123.39, 124.55, 125.90, 127.93, 132.22, 135.86, 145.57, 147.27, 153.37, 154.67, 159.53, 161.50, 166.45. IR (KBr, cm^{-1}): 3449 (OH), 1702 (C=O), 1628, 1549, 1219, 1248, 740, 674. MS (EI) *m/z* ($C_{18}H_{10}FNO_3$): 309 (M+2, 2.57%), 307 (M^+, 100%), 234 (15.94%), 160.08 (34%), 145.07 (68.05%), 89.08 (70.49%). Elemental Analysis for $C_{18}H_{10}FNO_3$, Calcd: C, 70.36; H, 3.28; N, 4.56; Found: C, 70.35; H, 3.27; N, 4.57.

6.1.8. Synthesis of 5-(2-benzofuran-2-yl)-6-fluoroquinolin-1,3,4-thiadiazol-2-amine (12)

To a mixture of compound **11** (0.2 g, 0.65 mmol) and $POCl_3$(15 ml), thiosemicarbazide (0.3 g, 3.5 mmol) was added and the mixture was refluxed and stirred for 48 h. The reaction mixture was cooled to rt, poured onto crushed ice with vigorous stirring, neutralized with an aqueous solution of KOH till pH = 12 and the separated solid was filtrated, washed with water and dried to give the titled compound as brown solids with m.p. = > 300 °C (0.225 g, yield = 98%).^{1}H-NMR (500 MHz, DMSO-d_6): δ = 7.79 (d, *J* = 9.5 Hz, 2H, NH_2), 7.81 (s, 3H, ArH), 7.83 (s, 3H, ArH), 7.93 (s, 2H, ArH), 7.96 (s, 1H, ArH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 107.02, 109.75, 110.02, 111.44, 118.46, 120.30, 120.59, 121.89, 123.38, 123.56, 125.84, 127.94, 132.31, 135.03, 145.54, 147.37, 153.31, 153.47, 154.64, 159.66, 161.61, 169.45.IR (KBr, cm^{-1}): 3748, 3446 (NH_2), 3421, 1628, 1506, 1244, 821, 744; %). Elemental Analysis for $C_{19}H_{11}FN_4OS$, Calcd: C, 62.97; H, 3.06; N, 15.46; Found: C, 62.96; H, 3.05; N, 15.47.

6.2. Biology

6.2.1. Cytotoxicity screening

In vitro cytotoxicity of all prepared compounds were tested by using a standard colorimetric method measuring a tetrazolium salt reduction via mitochondrial dehydrogenase activity (MTT assay) against a panel of five human tumor cell lines including hepatocellular carcinoma (HEPG-2), breast cancer (MCF-7), colorectal carcinoma (HCT-116), cervical carcinoma (Hela) and human prostate cancer (PC-3) obtained from ATCC *via* Holding Company for biological products and vaccines (VACSERA), Cairo, Egypt. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Penicillin and streptomycin were added at 37 ° C. Cells were seed in a 96-well plate.at 37° C for 48 h. After incubation, the cells were treated with different concentration of compounds and incubated for 24 h. After 24 h of drug treatment, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. 100 µl of dimethyl sulfoxide (DMSO) was added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X 100 (T.Mosmann and Immunol 1983).

6.2.2. Enzyme activity inhibition assay

6.2.2.1 Topoisomerase 1 inhibition assay

The eia Top 1 kit was based on ELISA technology. A 96-well plate was pre-coated by anti-Topo1 antibody which is a detection antibody. The standards, test samples and biotin conjugated detection antibody were added to the well. Then, HRP-Streptavidin was added and TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the Top 1 amount of sample captured in plate. Read the O.D. absorbance at 450 nm in a microplate reader, and then the concentration of Topo1 can be calculated. The assay procedure summarized that 100 µl of sample was added to each well and incubated for 90 minutes at 37 °C. Then, 100 µl of Biotin- detection antibody was added to each well and incubated for 60 minutes at 37 °C. Then, 100 µl of HRP-Streptavidin was added to each well and incubated for 30 minutes at 37 °C. 90 µL of TMB substrate was added and the wells were incubated for 15 -30 minutes at 37 °C. Adding 50 µl of Stop solution into each well and the

color changes into yellow. Optical density was measured by plate reader and the absorbance of each well was read at 450 nm. Using standard curve, the concentrations of topo1 can be calculated.

6.2.2.2 Aromatase inhibition assay

The kit is a sandwich enzyme immunoassay technique for the in vitro quantitative measurement of aromatase enzyme. The microtiter plate provided in this kit has been pre-coated with an antibody specific to aromatase. Samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to aromatase. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain aromatase biotin-conjugated antibody and enzyme conjugated Avidin will exhibit a change in color. The color change is measured spectrophotometrically at a wavelength of 450 nm ±10 nm. The concentration of aromatase in the samples is then determined by comparing the O.D. of the

samples to the standard curve. Assay procedure summarized as 100 µl of sample was added to each well and the well incubated at 37 °C for 2 hr. Next, 100 µl of Detection Reagent A was added and the well incubated for 1 hr at 37 °C, then 100 µl of Detection Reagent B was added and the well incubated for 30 minutes at 37 °C. Substrate solution (90 µl) was added to well and incubated for 15-25 minutes at 37 °C. Adding 50 µl of stop solution into each well and the color changes into yellow. Optical density was measured by plate reader and the absorbance of each well was read at 450 nm. Using standard curve, the concentrations of aromatase can be calculated.

6.2.3. Cell cycle analysis and induction of apoptosis

6.2.3.1. Flow cytometric analysis of cell-cycle distribuation

For flow cytometric analysis of DNA content, HCT-116 cells in exponential growth were treated with compound **6a** and incubated for 24 h. The cells were collected, centrifuged and fixed with ice cold ethanol (70%). The cells were then treated with buffer containing RNAse A and 0.1% Triton X-100, then stained with propidum iodide and compared with control which was treated with DMSO. DNA contents were measured by flow cytometry.

6.2.3.2. Analysis of cellular apoptosis

HCT-116 cells in exponential growth were treated with compounds **6a** and incubated for 24 h. After an incubation period, 1-5 x 10^5 cells were harvested and suspended in 500 µl of 1X Binding Buffer, 5 µl of Annexin V-FITC and 5 µl of propidium iodide. They are incubated at room temperature for 5 min in the dark and incubated at 37 °C. Annexin V-FITC conjugate is specially engineered to produce enhanced fluorescence signal and photo stability. The Annexin V-FITC kit includes Annexin V-FITC for detecting apoptosis and propidium iodide (PI) for detecting necrosis. Thus, Apoptosis and necrosis can be differentiated. Detection and analyzing of Annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2). http://www.biovision.com.

6.3. Docking study

Molecular modeling calculations and docking studies were carried out using molecular operating environment (MOE) software version 2014.09 (Chemical Computing Group Inc., Montreal, Quebec, Canada). The protein data bank file (PDB ID: **1K4T**) was selected from this purpose http://www.rscb.org. The file contain the crystal structure of Topo1 with Topotecan was obtained from protein data bank. All water molecules in PDB were ignored and hydrogen atoms were added to the protein, the energy minimized using MMFF94x force field and the conformers generated were docked into the Top1 receptor with MOE-DOCK using the triangle matcher placement method and the London dGscoring function. The validated docking protocol in the active site was then used to study the ligand-receptor interactions for the novel hybrids to predict their binding mode and binding affinity.

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