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Preliminary communication

Regioselective synthesis of polycyclic aza-oxa and aza-oxa-thia heteroarenes as Colo-205 and HepG2 carcinoma cells growth inhibitors

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ABSTRACT

An efficient regioselective synthesis of polycyclic diheteroaryl[*b*,*d*]pyrans and diheteroaryl[*c*,*e*][1,2]diazepines has been reported through ring transformation reactions of 2-oxo-2,5-dihydrothiochromeno[4,3-*b*]pyrans (**3**,**4**), 2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine/thiepine (**8**, **9**) and 6-oxo-3,6-dihydro-2*H*-naphtho[1,2-*b*]pyrano[2,3-*d*]oxepine (**15**) by hydrazine, at ambient and reflux temperature. Nine compounds viz **5a**,**b**; **10a**,**c**,**d**; **12b**; **13b**; **16** and 1-methylthio-5,6-dihydrobenzo[*f*]quinoline (0.1–100 μM) were screened for their cytotoxicity in normal (IEC-6), carcinoma (Colo-205) and HepG2 cell lines. None of the compounds showed cytotoxicity in normal IEC-6 cells while **10a**,**d** and **16** resulted in killing of Colo-205 cells with IC_{50} ranging 20–60 μM while **10c** and **13b** caused killing of HepG2 cells with IC_{50} values ranging 60–80 μM concentration. Further, **10a**,**d** and **16** caused apoptosis through a cascade of mitochondrial pathway in Colo-205 cells indicating anticancerous potential against intestinal cancer. Interestingly, compounds **10c** and **13b** exhibited apoptosis through mitochondrial pathway in HepG2 cells suggesting anticancer activity against hepatic cancer.

1. Introduction

Cancer is a leading cause of death, accounting for 8.2 million deaths worldwide in 2012. Colorectal and malignant hepatoma are the most common cancer of colon and liver respectively [1,2]. It is estimated that more than one million people get colorectal cancer yearly worldwide [3], resulting in about half million deaths [4]. It is estimated second most common in women and the third most common cancer in men [5]. While hepatocellular carcinoma causes 662,000 deaths worldwide per year [6]. Overall, liver cancer have the third and colon cancer have the fourth most common cause of cancer death after lung and stomach cancer [7]. Due to lack of efficacious and economical drugs without adverse side effects, it needs continuous efforts to synthesize and study anticancer efficacy of new heterocycles.

Mollugin (**I**), isolated from the Chinese medicinal plant *Rubia cardifolia* [8] has demonstrated potential antitumor, antimutagenic and antiviral activity against hepatitis-B virus [9]. A new compound tanshinlactone A **(II)**, also a natural product, isolated [10] from an ethanol extract of *Salvia miltiorrhiza* displayed cytotoxic activity [10] with CD_{50} range of 6.87–8.85 μg/ml against the Hela (cervical epitheloid carcinoma) and HepG2 (Hepatocellular carcinoma); Fig. 1.

The diverse pharmacological activity of compounds **I** and **II** (Fig. 1) prompted us to synthesize tetra- and pentacyclic diheteroaryl[*b*,*d*]pyrans (**5**, **10**) and diheteroaryl[*c*,*e*][1,2]diazepines (**12**, **13**) to assess the contribution of hetero atoms on pharmacological profiles for treatment of colon carcinoma and hepatocarcinoma, which are the third and fourth most frequently diagnosed cancers globally using Colo-205 and HepG2 cell lines, respectively.

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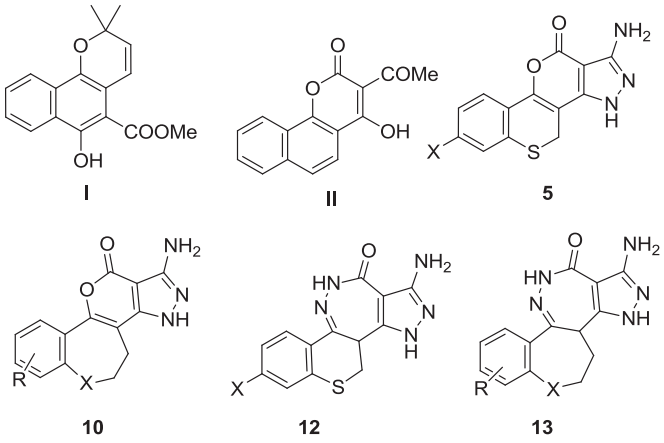


Fig. 1. Natural products **I**, **II** and various synthesized compounds **5**, **10**, **12** and **13**.

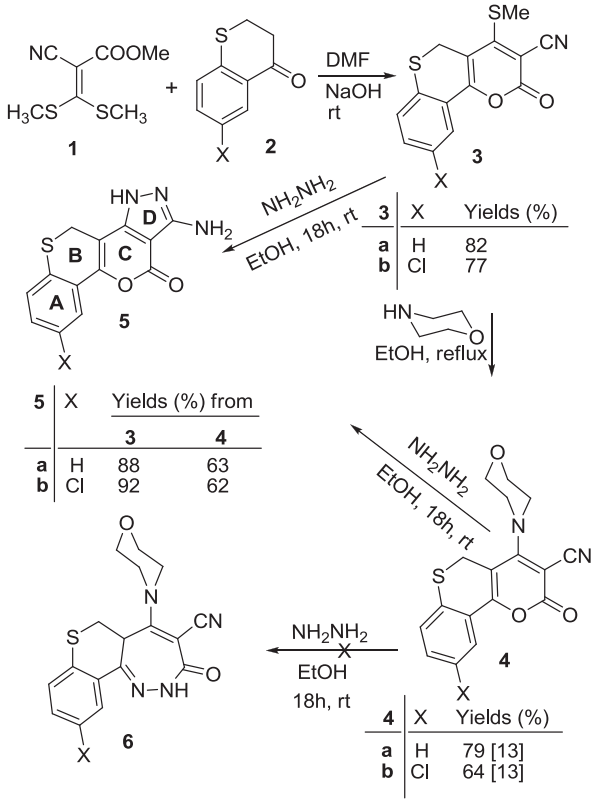
2. Results and discussion

2.1. Chemistry

Herein, we report an elegant regioselective synthesis of fused tetra-, and pentacyclic heteroarenes. Our primary synthetic strategy to make aza-oxa-, aza-thia- and aza-oxa-thiaheterocyles was based on the base catalyzed ring transformation of appropriate lactones (**3**, **4**, **8**, **9**, **15**). Precursor 4-methylthio-2-oxo-2,5-dihydrothiochromeno[4,3-*b*]pyran-3-carbonitriles (**3**) [13] were synthesized from the reaction of thiochroman-4-ones (**2**) with methyl 2-cyano-3,3-dimethylthioacrylate (**1**) [11,12]. Further reaction of precursor **3** with hydrazine hydrate (98%) produced 3-amino-4,11-dihydro-4-oxo-1*H*-pyrazolo[4,3-*c*]thiochromeno[3,4-*e*]pyrans (**5**) at room temperature, regioselectively (Scheme 1). The cyclic aminated product 4-morpholino-2-oxo-2,5-dihydrothiochromeno[4,3-*c*]pyran-3-carbonitrile (**4**) [13] was synthesized through amination of 4-methylthio-2-oxo-2,5-dihydrothiochromeno[4,3-*b*]pyran-3-carbonitriles (**3**) with morpholine in boiling ethanol. The aminated lactone (**4**) on further reaction with hydrazine (98%) under analogous reaction conditions produced the same compound **5**. Our rigorous attempts to prepare the anticipated ring transformed product (1*E*,4*E*)-5-morpholino-3-oxo-2,3,5a,6-tetrahydrothiochromeno[4,3-*c*][1,2]diazepine-4-carbonitrile (**6**) failed, Scheme 1.

To ensure the generality of this reaction, precursors **8** and **9** were used for the construction of 'Z' shaped aza-oxa- (**10a**–**d**) and aza-oxa-thia heteroarenes (**10e**). The precursor (**8**) [14,15] was obtained from the reaction of benzo[*b*]oxepin/thiepin-5-one with methyl 2-cyano-3,3-dimethylthioacrylate (**1**). Amination of **8** with *sec*.amine in refluxing methanol afforded 4-*sec*.amino-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine-3-carbonitriles (**9**) [14,15]. Further, reaction of **8** or **9** with hydrazine (98%) in methanol at room temperature led to yield a product 3-amino-4,11,12-trihydro-4-oxo-1*H*-pyrazolo[4,3-*c*]benzo[*b*]oxepino[4,5-*e*]pyrans (**10**) in lieu of anticipated tricyclic 2,5a,6,7-tetrahydrobenzo[*b*]oxepino[1,2]diazepine (**11**) possibly due to steric factor, Scheme 2. The structure of **10e** has been also confirmed by X-ray crystallography, Fig. 2 [16].

The molecular make up of **8** and **9** revealed the presence of three electrophilic sites C-2, C-4 and C-11b in which later is more electron deficient due to extended conjugation and the presence of electron withdrawing substituent at position 3 of the lactone ring. Thus, C-11b position seems susceptible to nucleophilic attack but practically reaction takes place at C-4 position with formation of Michael adduct followed by condensation-cyclization to yield **10**. Possibly, the intramolecular C—H···O interaction [17] between nuclear oxygen and ortho hydrogen of aryl ring plays significant role in

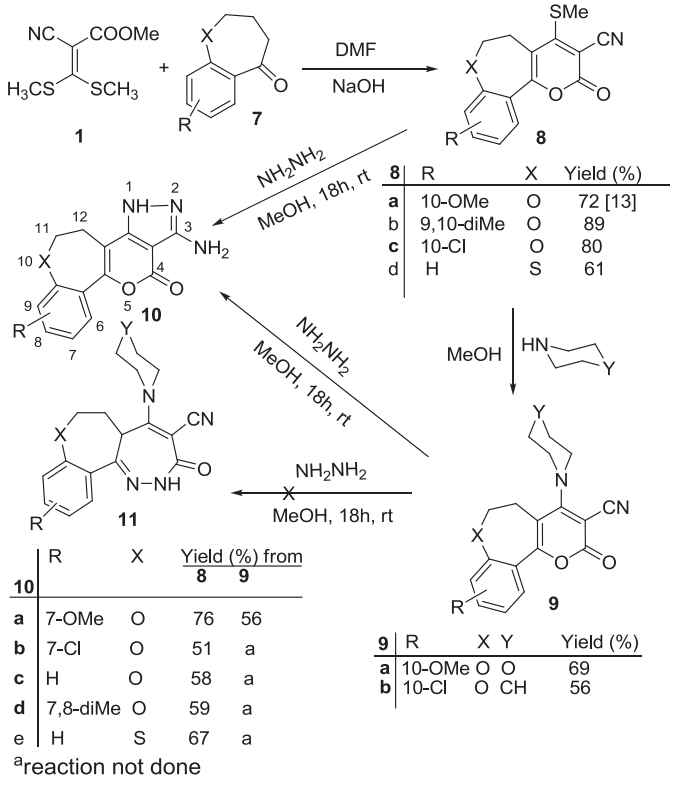


3	X	Yields (%)
a	H	82
b	Cl	77

5	X	Yields (%) from 3	Yields (%) from 4
a	H	88	63
b	Cl	92	62

4	X	Yields (%)
a	H	79 [13]
b	Cl	64 [13]

Scheme 1. Synthesis of 'Z' shaped diheteroaryl[*c*,*e*]pyrans (**5**).



8	R	X	Yield (%)
a	10-OMe	O	72 [13]
b	9,10-diMe	O	89
c	10-Cl	O	80
d	H	S	61

10	R	X	Yield (%) from 8	Yield (%) from 9
a	7-OMe	O	76	56
b	7-Cl	O	51	a
c	H	O	58	a
d	7,8-diMe	O	59	a
e	H	S	67	a

[a]reaction not done

9	R	X	Y	Yield (%)
a	10-OMe	O	O	69
b	10-Cl	O	CH	56

Scheme 2. Synthesis of 'Z' shaped aza-oxa-thia tetracyclic diheteroaryl[*c*,*e*]pyrans (**10**).

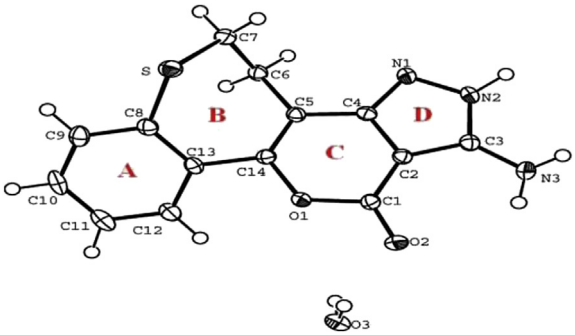
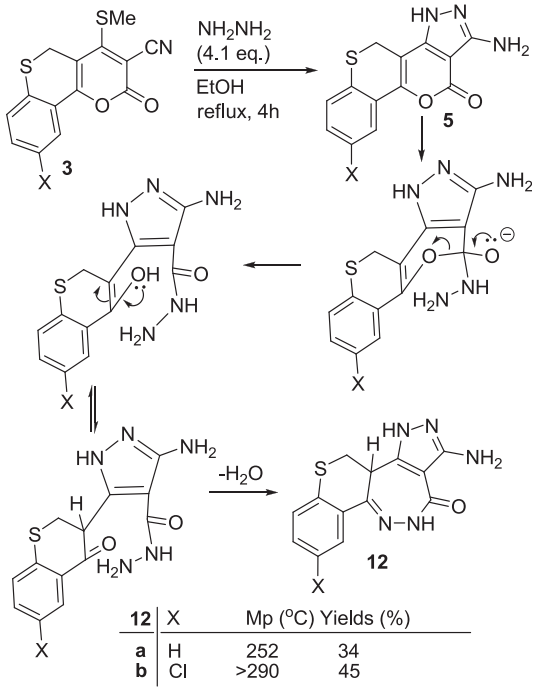


Fig. 2. ORTEP diagram of molecule of **10e** at 50% probability with atom numbering [16].

facilitating the reaction at C-4 site. A plausible reaction mechanism of the reaction is shown in Fig. 3.

In our efforts to synthesize 'Z' shaped aza-thia tetracyclic diheteroaryl[*c,e*][1,2]diazepines (**12**), we explored the reaction of 4-methylthio-2-oxo-2,5-dihydrothiochromeno[4,3-*b*]pyran-3-carbonitriles (**3**) with hydrazine (98%) in boiling ethanol which led to yield a product different from **10** and was characterized as 3-amino-5,12,12a-trihydro-4-oxo-1*H*-pyrazolo[4,3-*e*]thiochromeno [4,3-*c*][1,2]diazepines (**12**), Scheme 3. The initial step in this reaction is possibly due to the formation of **5** as an intermediate, which



Scheme 3. Synthesis of 'Z' shaped aza-thiatetracyclic diheteroaryl[*c,e*][1,2]diazepines (**12**).

on further reaction with excess hydrazine hydrate at elevated temperature resulted **12** through ring opening followed by cyclization. Further, isolation of product **12** directly from the reaction of **5** with hydrazine hydrate at reflux temperature proved the reaction pathway through intermediary of **5**.

To explore the generality of this reaction, we further explored the reaction of **8** with hydrazine hydrate (98%) in boiling ethanol which yielded a mixture of products **10** and **13**. However, this reaction in methanol and DMF (15%) gave regioselectively tetracyclic

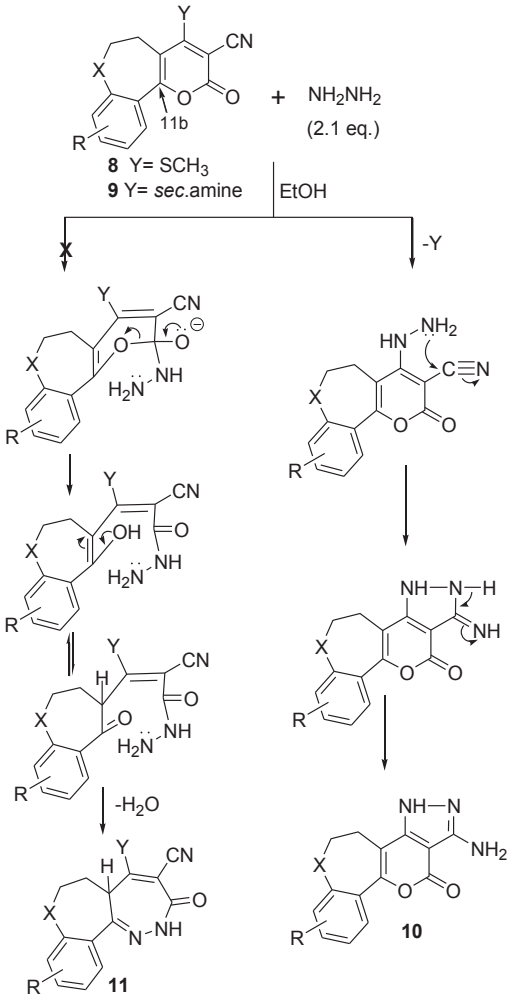
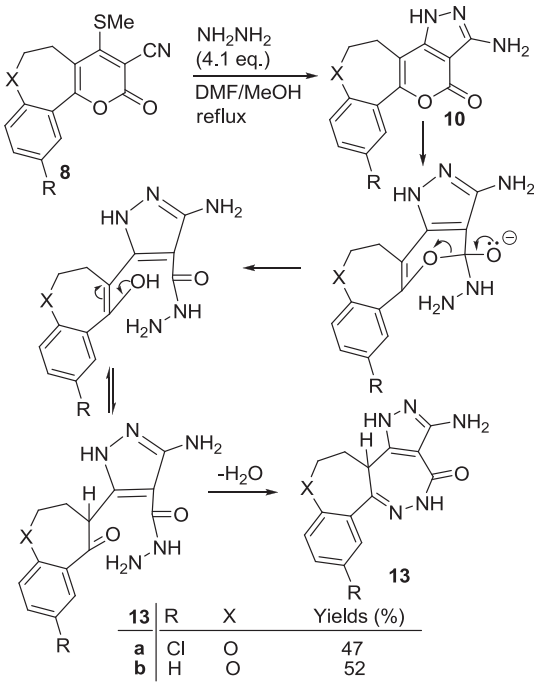
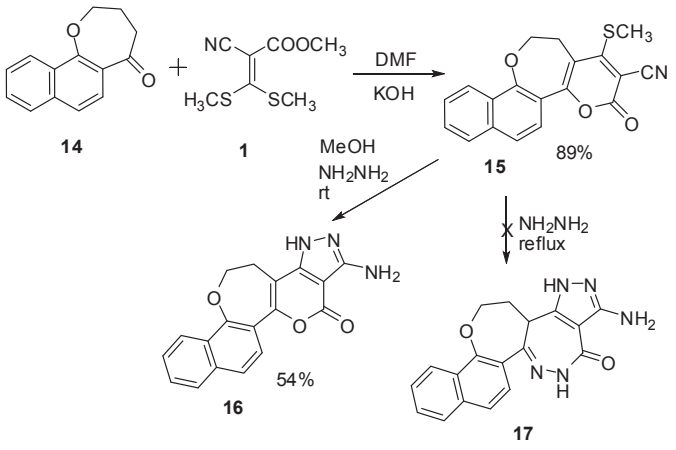


Fig. 3. A plausible reaction mechanism for the synthesis of "Z" shaped diheteroaryl[*c,e*] pyrans (**10**).



Scheme 4. Synthesis of 'Z' shaped tetrahydro-1*H*-pyrazolo[3,4-*e*] benzo[*b*]oxepino [5,4-*c*]][1,2]diazepines (**13**).



Scheme 5. Synthesis of pentacyclic aza-oxa diheteroaryl[*c*,*e*][1,2]diazepine (**16**).

diheteroaryl[*c*,*e*][1,2]diazepines (**13**), Scheme 4. The initial step in this reaction was the formation of **10** as an intermediate, which in the presence of excess of hydrazine hydrate at reflux temperature produced the product **13**. It was further directly isolated from the reaction of **10** with hydrazine hydrate that proved the intermediary of **10**.

Additionally, we have explored the reaction of 4-methylthio-2-oxo-5,6-dihydro-2*H*-naphtho[*b*]oxepino[5,4-*b*]pyran-3-carbonitrile (**15**), obtainable from the reaction of 3,4-dihydronaphtho[1,2-*b*]oxepin-5(2*H*)-one (**14**) with methyl 2-cyano-3,3-bismethylthioacrylate (**1**) in DMF with hydrazine at room temperature in methanol which exclusively gave pentacyclic diheteroaryl[*c*,*e*][1,2]diazepine (**16**). However, reaction in a mixture

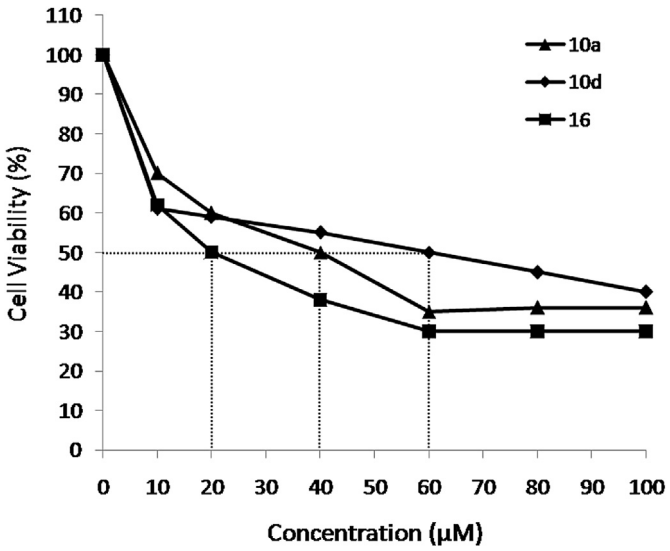


Fig. 5. IC_{50} values of compounds **10a**, **10d** and **16** in Colo 205 cells.

of refluxing methanol and DMF (15%) at reflux temperature gave a complex mixture in lieu of anticipated pentacyclic diheteroaryl[*c*,*e*][1,2]diazepine (**17**), Scheme 5.

Thus, we have explored ring transformation reactions as one of the most powerful protocols for the synthesis of polycyclic heteroarenes. 4-Substituted-2-oxo-2,5-dihydrothiochromeno[4,3-*b*]pyran-3-carbonitriles (**3**, **4**), 4-substituted-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine/thiepine-3-carbonitriles (**8**, **9**) and 4-methylthio-2-oxo-5,6-dihydro-2*H*-naphtho[*b*]oxepino[5,4-*b*]

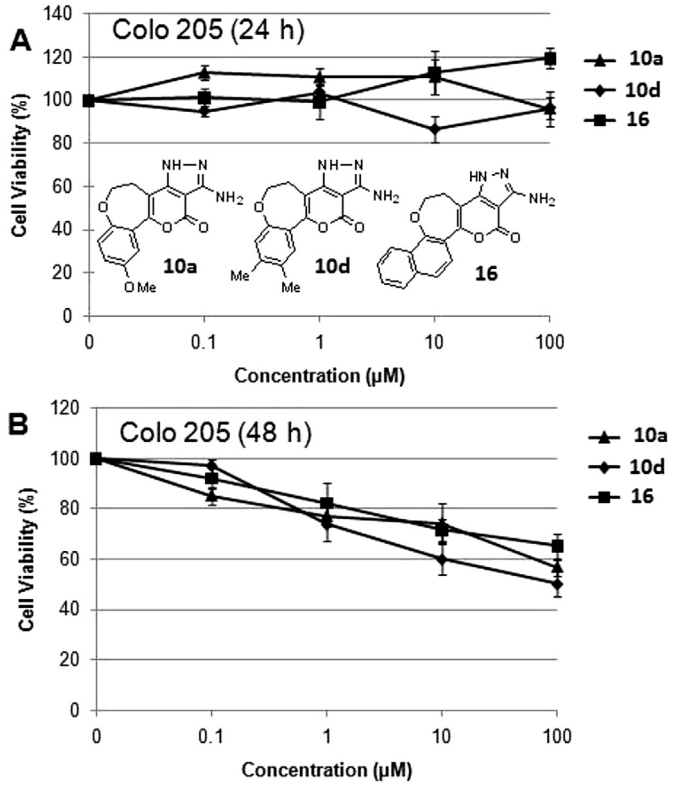


Fig. 4. Inhibitory effect of the compounds **10a**, **10d** and **16** on the cell viability of cancerous cells Colo 205.

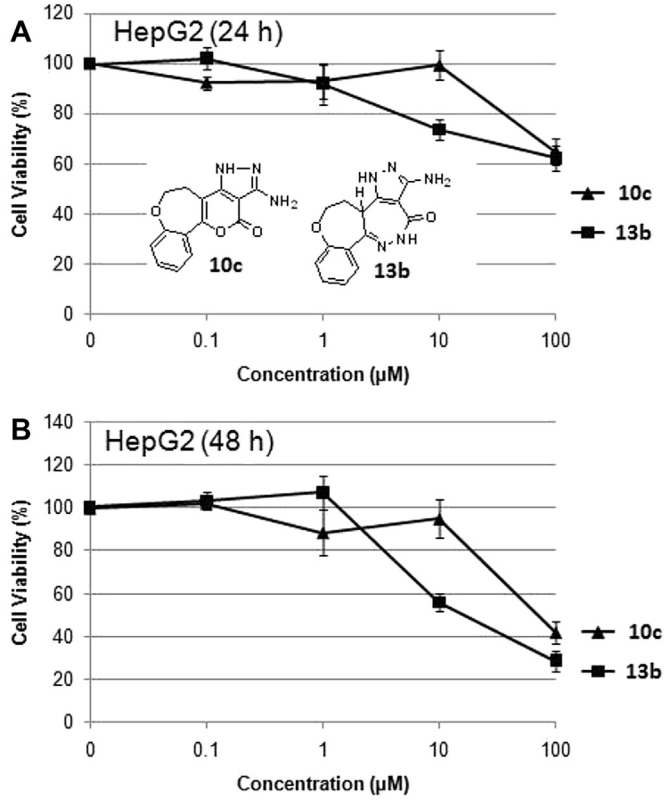


Fig. 6. Inhibitory effect of the compounds **10c** and **13b** on the cell viability of cancerous cells HepG2.

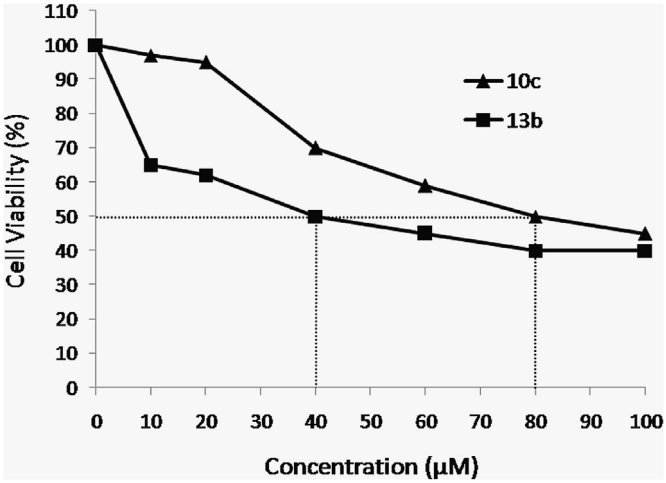


Fig. 7. IC_{50} values of compounds **10c** and **13b** in HepG2 cells.

pyran-3-carbonitrile (**15**) were found excellent substrates for the ring transformation reactions. These substrates behave as the synthetic equivalent of cyclic ketene hemithioacetal, useful for building various four and five membered fused heterocycles ring skeleton by the reaction with ambiphilic nucleophile.

2.2. Cytotoxicity study

Cytotoxicity of the synthesized products **5a**, **b**, **10a**,**c**,**d**, **12b**, **13b**, **16** and 1-methylthio-5,6-dihydrobenzo[*f*]quinoline [18] were studied by MTT assay [19] in IEC-6 (normal), Colo-205 and HepG 2 (carcinoma) cell lines and the results are shown below.

2.2.1. Human intestinal epithelial cells (IEC-6)

The effect of **5a**,**b**, **10a**,**c**,**d**, **12b**, **13b**, **16** and 1-methylthio-5,6-dihydrobenzo[*f*]quinoline [18] showed no cytotoxicity in IEC-6 (normal) cells following 24 and 48 h of incubation indicating that these compounds are safe for normal cells upto a maximum concentration of 100 μM (Supplementary Fig. 1).

2.2.2. Human colorectal carcinoma cells (Colo-205)

Of the nine synthetic compounds, only **10a**, **10d** and **16** showed dose dependent cytotoxicity at 48 h while no cytotoxic effects were observed at 24 h in Colo-205 cells (Fig. 4). In one of the experiments, a 10 fold higher dose was used for each synthetic compound to determine 50% inhibition of cytotoxicity (IC_{50}) value at 48 h. Fig. 5 showed the IC_{50} value for **10a**, **10d** and **16** in Colo-205 cells at 40, 60 and 20 μM concentration respectively at 48 h.

2.2.3. Human hepatocellular carcinoma cells (HepG2)

Synthetic compounds **5a**,**b**; **12b**; **10a**,**d** and 1-methylthio-5,6-dihydrobenzo[*f*]quinoline [18] displayed no cytotoxicity upto

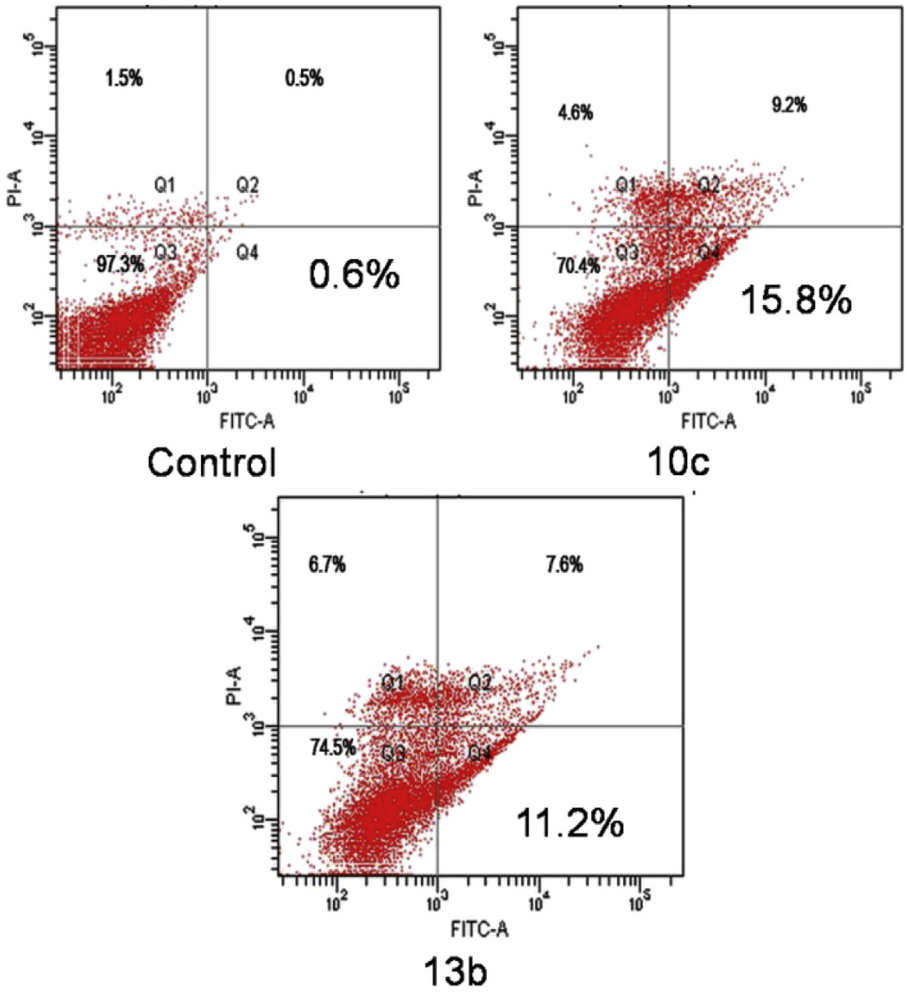


Fig. 8. Apoptotic analysis of HepG2 cells treated with **10c** and **13b** after 48 h exposure.

100 μM concentration in HepG2 cells at 24 and 48 h. However, compounds **10c** and **13b** (0.1–100 μM) showed significant cytotoxicity in a dose dependent manner at 24 and 48 h (Fig. 6). The IC_{50} values of **10c** and **13b** in HepG2 cells were found to be 80 and 60 μM, respectively, at 48 h (Fig. 7).

2.2.4. Apoptosis in HepG2 and Colo-205 cells

The apoptotic effects of **10c** and **13b** at IC_{50} were undertaken in HepG2 cells at 48 h (Fig. 8). The synthesized compounds **10c** and **13b** enhanced apoptosis by 15.8 and 11.2% compared to untreated HepG2 cells (0.6%) (Fig. 8). Fig. 9 depicts the effect of **10a**,**d** and **16** on apoptosis in Colo-205 cells following 48 h of treatment. It was observed that **10a**,**d** and **16** significantly enhanced necrosis (3–7.7%) compared to untreated Colo-205 cells (0.5%), suggesting that these cancerous cells had high proliferative rate thereby causing the cells to undergo necrosis and did not show any substantial effect on apoptosis (Fig. 9). Hence, experiments related to apoptosis in Colo-205 cells were carried out at 24 h. Synthesized compounds, **10a**, **10d** and **16** resulted in early apoptosis by 16.7, 9.4 and 5.5% compared to untreated Colo-205 cells (0.07%) (Fig. 10).

2.2.5. Apoptosis related proteins in HepG2 and Colo-205 cells

Effect of synthesized compounds **10c** and **13b** on apoptosis related proteins in HepG2 cells is shown in Fig. 11. The major tumor suppressor protein p53 is of wild type in HepG2 cells and due to its important role in cell cycle regulation and apoptosis; its expression level was analyzed following **10c** and **13b** treatment. The result showed that p53 protein along with its target protein p21, an important cell cycle regulatory protein, was over expressed in HepG2 cells. Furthermore, treatment of HepG2 cells by **10c** and **13b** for 48 h resulted in overexpression of pro-apoptotic protein, Bax and suppression of anti-apoptotic protein, Bcl2 as compared to vehicle treated control. Cytochrome C was also found to be enhanced in HepG2 cells as compared to the control which further leads to the activation of pro-caspases 9 and 3 which was revealed by enhancement in the levels of cleaved caspases 9 and 3 following **10c** and **13b** treatment. Further, activated caspase 3 led to the cleavage of PARP, a DNA repair enzyme, in the treated HepG2 cells whereas in control cells intact band of PARP at 116 kDa was observed. Thus, the above results indicate that **10c** and **13b** treatment led to the significant apoptosis in hepatocellular carcinoma HepG2 cells by modulating p53 dependent pathway (Fig. 11).

Effect of compounds **10a**, **10d** and **16** on apoptosis related proteins in Colo-205 cells is shown in Fig. 12. Since Colo-205 lack p53 protein, **10a**, **10d** and **16** showed no effect in Colo-205 cells. These three compounds showed overexpression of p21/waf1, a cell cycle regulatory protein, suggesting that cell cycle arrest may be possible in a p53 independent manner in Colo-205 cells. Bax, a pro-apoptotic protein, was found to be enhanced by **10a**, **10d** and **16** while Bcl2, a potent anti-apoptotic protein was found to be decreased by these compounds. The observed increase in Bax/Bcl2 ratio acts as proapoptotic signal resulting in the release of

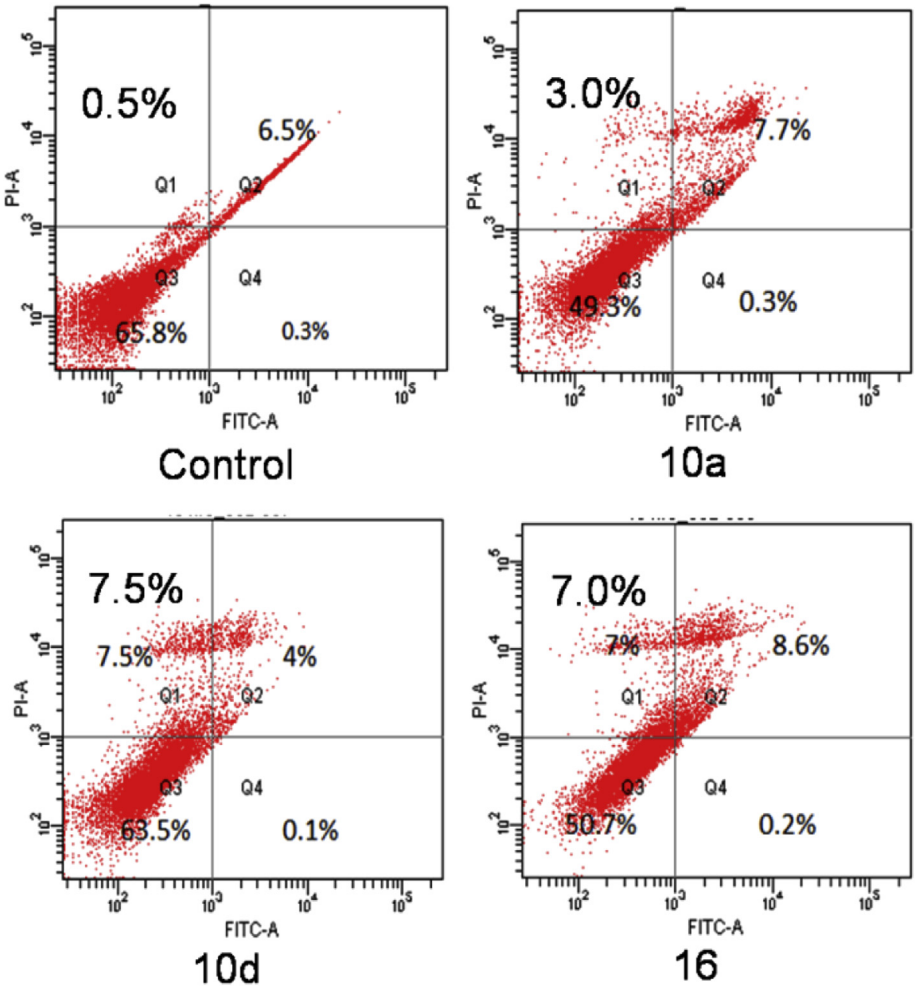


Fig. 9. Apoptotic analysis of Colo-205 cells treated with **10a**, **10d** and **16** after 48 h exposure.

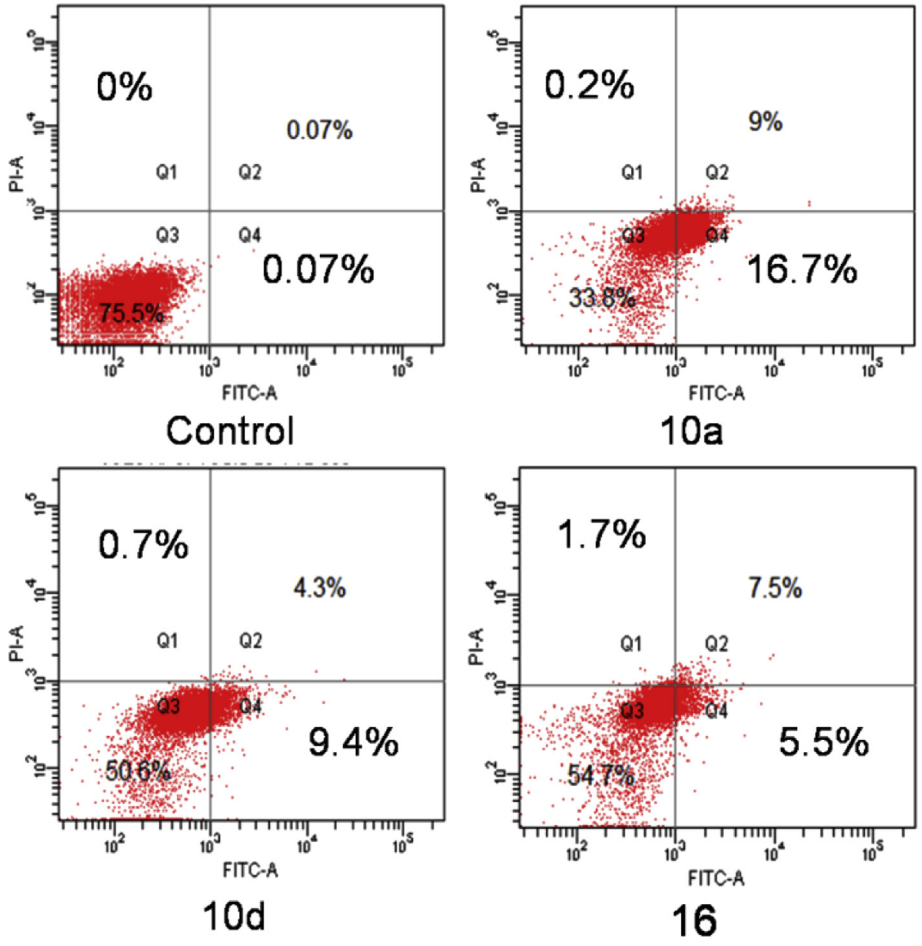


Fig. 10. Apoptotic analysis of Colo-205 cells treated with **10a**, **10d** and **16** after 24 h exposure.

cytochrome C from mitochondria into cytoplasm as revealed in Fig. 12. Released cytochrome C leads to the formation of apoptosome which in turn cause activation of caspase 9, thereby cleaving procaspase 3 to its activated form caspase 3 resulting in the cleavage of PARP protein, resulting in DNA degradation and apoptotic cell death as observed in Fig. 12. Since, caspase 8 involved in extrinsic cell death, was not found to be activated by **10a**,**d** and **16** (data not shown), the apoptosis in Colo-205 cells by these compounds in related to mitochondrial cell death.

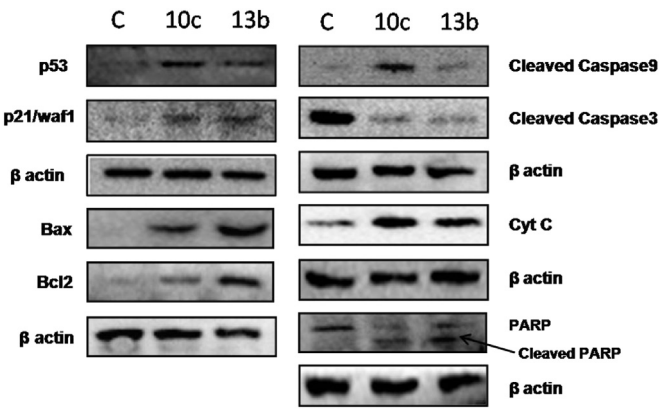


Fig. 11. Effect of **10c** and **13b** on apoptosis related proteins in HepG2 cells.

Structure activity relationship showing that sulfur atom and six membered ring B and lactone part in ring C (**5**, Scheme 1) are playing crucial role in apoptosis in comparison with oxygen atom and seven membered ring B and/or hydrazine part in ring C (**10a**, **10c**, **10d**, **13b**, **16**, Schemes 2–5).

3. Conclusions

In summary, we have developed an efficient non-catalytic route for the construction of 'Z' shaped partially reduced diheteroaryl [*b*,*d*]pyrans and diheteroaryl[*c*,*e*][1,2]diazepines by base induced inter- and intramolecular C–N bond formation from the reaction of 4-substituted-2-oxo-2,5-dihydrothiochro- meno[4,3-*b*]pyran-3-

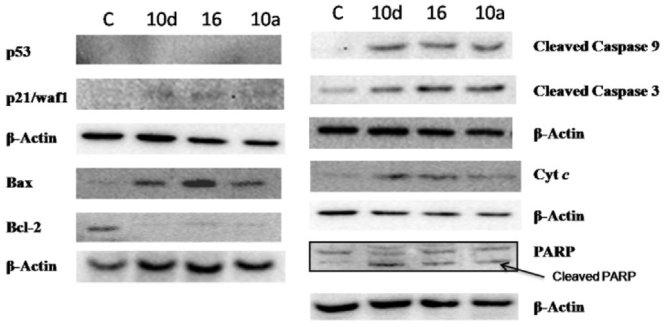


Fig. 12. Effect of **10a**, **10d** and **16** on apoptosis related proteins in Colo-205 cells.

carbonitriles (**3**,**4**), 4-substituted-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine/thiepine-3-carbonitriles (**8**) and 4-methylthio-2-oxo-5,6-dihydro-2*H*-naphtho[*b*]oxepino[5,4-*b*]pyran-3-carbonitrile (**15**) by hydrazine at room and reflux temperature separately. The protocol is highly flexible and compatible to functional groups for the construction of desired shape of partially reduced polycyclic heteroarenes. The effect of synthesized compounds on the carcinoma cells Colo-205 and HepG2 without any cytotoxicity in IEC-6 cells (normal) indicate that **10c** and **13b** treatment led to significantly enhance apoptosis in hepatocellular carcinoma HepG2 cells, while **10a**,**d** and **16** caused apoptosis in Colo-205 cells by modulating p53 dependent pathway and that these compounds may be a potential candidate for the treatment of hepatic and colorectal cancers.

4. Experimental

4.1. Materials and methods

The reagents and the solvents used in this study were of analytical grade and were used without further purification. The melting points were determined on an electrically heated Townson Mercer melting point apparatus and are uncorrected. Commercial reagents were used without purification. ^{1}H and ^{13}C NMR spectra were measured on a Bruker WM-300 (300 MHz)/Jeol-400 using $CDCl_3$ and DMSO-d_6 as the solvents. Chemical shift are reported in parts per million shift (δ-value) from Me_4Si (δ 0 ppm for ^{1}H NMR) or based on the middle peak of the solvent ($CDCl_3$) (δ 77.00 ppm for ^{13}C NMR) as the internal standard. Signal patterns are indicated as s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; bh, broad hump. Coupling constant (J) are given in Hertz. Infrared (IR) spectra were recorded on a Perkin–Elmer AX-1 spectrophotometer in KBr disc and reported in wave number (cm^{-1}). ESIMS spectrometers were used for mass spectra analysis. ^{13}C NMR spectra of all compounds were not reported due to their very poor solubility in deuterated solvents (DMSO-d_6 and $CDCl_3$).

4.2. General procedure for the synthesis of 4-methylthio-2-oxo-2,5-dihydrothiochromeno[4,3-b]pyran-3-carbonitriles (*3*)

A mixture of thiochroman-4-ones (**2**, 5 mmol) and methyl 2-cyano-3,3-dimethylthioacrylate (**1**, 5 mmol) in DMF (8 mL) was stirred in the presence of powdered NaOH (7 mmol) for 14 h, at room temperature. The reaction mixture was poured onto crushed ice with vigorous stirring. The aqueous suspension was neutralized with 10% HCl and the precipitate obtained was filtered, washed with water, dried and crystallized from acetone.

4.2.1. 4-Methylthio-2-oxo-2,5-dihydrothiochromeno[4,3-b]pyran-3-carbonitrile (*3a*)

Canary yellow amorphous solid; mp 179–180 °C; IR (KBr): 2163 (CN), 1682 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.95 (s, 3H, CH_3), 4.06 (s, 2H, SCH_2), 7.36 (d, 1H, J = 3.6 Hz, Ar–H), 7.47 (d, 2H, J = 3.6 Hz, Ar–H), 7.80 (d, 1H, J = 8.0 Hz, Ar–H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.3, 23.1, 94.5, 109.1, 114.4, 125.4, 126.1(2C), 127.2, 132.0, 135.9, 153.3, 156.8, 166.7; m/z (ESI): 287 (M^+); HRMS (ESI): M^+ calcd for $C_{14}H_9NO_2S_2$ 287.0075, found 287.0084.

4.2.2. 9-Chloro-4-methylthio-2-oxo-2,5-dihydrothiochromeno[4,3-b]pyran-3-carbonitrile (*3b*)

Canary yellow amorphous solid; mp 192–194 °C; IR (KBr): 2214 (CN), 1721 (C=O) cm^{-1}; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 2.94 (s, 3H, CH_3), 4.06 (s, 2H, SCH_2), 7.50 (m, 2H, Ar–H), 7.72 (m, 1H, Ar–H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.7, 23.5, 95.4, 110.2, 114.7, 125.9, 127.3, 129.2, 130.9, 131.8, 135.3, 152.2, 157.0, 166.9; m/z (ESI): 321 (M^+); HRMS (ESI): MH^+ calcd for $C_{14}H_9ClNO_2S_2$ 321.9763, found 321.9777.

4.3. General procedure for the synthesis of 4-sec.amino-2-oxo-2,5-dihydrothiochromeno[4,3-b]pyran-3-carbonitriles (*4*)

A mixture of 4-methylthio-2-oxo-2,5-dihydrothiochromeno [4,3-*b*]pyran-3-carbonitrile (**3**, 1 mmol) and *sec*.amine (1.1 mmol) was refluxed in absolute ethanol for 6 h. During this period a precipitate separated out which was filtered after cooling. The precipitate was washed with cold ethanol and finally crystallized with acetone.

4.3.1. 4-Morpholino-2-oxo-2,5-dihydrothiochromeno[4,3-b]pyran-3-carbonitrile (*4a*)

Orange colored crystalline solid; mp 216–218 °C; IR (KBr): 2212 (CN), 1714 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.68 (m, 4H, 2 × OCH_2), 3.76 (m, 4H, 2 × NCH_2), 3.89 (s, 2H, SCH_2), 7.32 (m, 1H, Ar–H), 7.43 (m, 2H, Ar–H), 7.74 (d, 1H, J = 7.8 Hz, Ar–H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.6, 51.7 (2C), 66.2 (2C), 79.9, 106.6, 116.6, 126.0, 126.4, 127.1, 127.4, 131.8, 135.8, 156.1, 160.0, 165.0; m/z (ESI): 326 (M^+); HRMS (ESI): MH^+ calcd for $C_{17}H_{15}N_2O_3S$ 327.0803, found 327.0810.

4.3.2. 9-Chloro-4-morpholino-2-oxo-2,5-dihydrothiochromeno [4,3-b] pyran-3-carbonitrile (*4b*)

Orange colored crystalline solid; mp 205–208 °C; IR (KBr): 2213 (CN), 1717 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.59 (bs, 4H, 2xOCH_2), 3.78 (bs, 4H, 2xNCH_2), 3.93 (s, 2H, SCH_2), 7.51 (bs, 2H, Ar–H), 7.71 (bs, 1H, Ar–H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.6, 51.8 (2C), 66.2 (2C), 80.3, 107.3, 116.5, 119.2, 125.3, 128.6, 129.2, 130.8, 131.3, 134.8, 154.8, 159.9, 164.7; m/z (ESI): 360 (M^+); HRMS (ESI): MH^+ calcd for $C_{17}H_{14}ClN_2O_3S$ 361.0414, found 361.0394.

4.4. General procedure for the synthesis of 3-amino-4,11-dihydro-4-oxo-1H-pyrazolo[4,3-c]thiochromeno[3,4-e]pyrans (*5*)

A mixture of 4-methylthio-2-oxo-2,5-dihydrothiochromeno [4,3-*b*]pyran-3-carbonitrile (**3**, 1 mmol) or 4-*sec*.amino-2-oxo-2,5-dihydro thiochromeno[4,3-*b*]pyran-3-carbonitrile (**4**, 1 mmol) and hydrazine (98%, 2.1 mmol) in ethanol was stirred at room temperature for 18 h. After completion, the reaction mixture was poured onto ice water with vigorous stirring and the precipitate obtained was filtered, washed with water and purified by crystallization from a mixture of 4% DMF in methanol.

4.4.1. 3-Amino-4,11-dihydro-4-oxo-1H-pyrazolo[4,3-c] thiochromeno[3, 4-e]pyran (*5a*)

Yellow solid; mp 290–292 °C; IR (KBr): 3445, 3247 (NH_2 & NH), 1692 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.98 (s, 2H, SCH_2), 6.46 (bh, 2H, NH_2); 7.27 (m, 2H, Ar–H), 7.31 (m, 1H, Ar–H), 7.68 (m, 1H, Ar–H); 12.46 (bh, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.6, 88.0, 103.3, 124.2, 126.0, 127.3, 127.6, 129.3, 129.3, 133.1, 147.6, 150.5, 157.9; m/z (ESI): 272 (MH^+); HRMS (ESI): MH^+ calcd for $C_{13}H_{10}N_3O_2S$ 272.0449, found 272.0412.

4.4.2. 3-Amino-7-chloro-4,11-dihydro-4-oxo-1H-pyrazolo[4,3-c] thiochromeno[3,4-e]pyran (*5b*)

Yellow solid; mp 300–302 °C; IR (KBr): 3445, 3260 (NH_2 & NH), 1687 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.00 (s, 2H, SCH_2), 6.59 (bh, 2H, NH_2); 7.34 (m, 1H, Ar–H), 7.38 (m, 1H, Ar–H), 7.60 (s, 1H, Ar–H); 12.47 (bh, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 21.6, 88.1, 103.4, 123.5, 126.1, 127.4, 128.9, 129.2, 130.5, 132.1,

147.8, 150.4, 157.5; *m/z* (ESI): 306 (MH^+); HRMS (ESI): MH^+ calcd for $C_{13}H_9ClN_3O_2S$ 306.0104, found 306.0115.

4.5. General procedure for the synthesis of 4-methylthio-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine/thiepine-3-carbonitriles (**8**)

A mixture of methyl 2-cyano-3,3-dimethylthioacrylate (**1**, 1 mmol) in DMF (8 mL) and 3,4-dihydro-2*H*-benzo[*b*]oxepin/thiepin-5(2*H*)-one (**7**, 1 mmol) was stirred in the presence of powdered NaOH (1.2 mmol) for 8 h and the reaction mixture was poured onto crushed ice with vigorous stirring. The aqueous suspension was neutralized with 5% HCl and the precipitate obtained was filtered, washed with water and finally crystallized with methanol.

4.5.1. 10-Methoxy-4-methylthio-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine-3-carbonitrile (**8a**)

Yellow amorphous solid; mp 178 °C; IR (KBr): 2216 (CN), 1722 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.87 (t, J = 6 Hz, 2H), 3.01 (s, 3H, SCH_3), 3.83 (s, 3H, OCH_3), 4.47 (t, 2H, J = 6 Hz, OCH_2), 7.03 (s, 1H, ArH), 7.28 (d, J = 9 Hz, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.1, 27.2, 55.9, 75.8, 93.7, 112.1 (2C), 114.5, 115.7, 120.6, 123.3, 124.1, 150.9, 155.8, 158.1, 168.3; *m/z* 315 (M^+); HRMS (ESI): M^+ calcd for $C_{16}H_{13}NO_4S$ 316.0626, found 316.0633.

4.5.2. 9,10-Dimethyl-4-methylthio-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine-3-carbonitrile (**8b**)

Yellow amorphous solid; mp 172 °C; IR (KBr): 2217 (CN), 1698 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.35 (s, 3H, Me), 2.43 (s, 3H, Me), 2.71 (t, J = 6 Hz, 2H), 3.02 (s, 3H, SCH_3), 4.43 (t, 2H, J = 6 Hz, OCH_2), 6.81 (s, 1H, ArH), 6.93 (s, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.8, 20.3, 21.3, 25.1, 77.8, 93.5, 114.6, 115.2120.8, 122.4, 128.6, 139.1, 143.6, 155.6, 157.9, 158.3, 167.4; *m/z* (ESI): 314 (MH^+); HRMS (ESI): M^+ calcd for $C_{17}H_{15}NO_3S$ 313.0773, found 313.0761.

4.5.3. 10-Chloro-4-methylthio-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine-3-carbonitrile (**8c**)

Light yellow solid; mp 200 °C; IR (KBr): 2218 (CN), 1713 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.90 (bs, 2H, CH_2), 2.92 (s, 3H, SCH_3), 4.45 (bs, 2H, OCH_2), 7.15 (m, 1H, Ar–H), 7.56 (m, 1H, Ar–H), 7.77 (m, 1H, Ar–H); ^{13}C NMR ($CDCl_3$): δ 18.0 27.8, 73.6, 123.3, 123.8, 123.9, 128.3, 132.5, 132.8, 132.9, 153.2, 156.2, 169.0; HRMS (ESI): M^+ calcd for $C_{15}H_{10}NO_3SCl$ 319.0370, found 319.0300.

4.5.4. 4-Methylthio-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]thiepine-3-carbonitrile (**8d**)

Yellow powder; mp 152 °C; IR (KBr): 2210 (CN), 1727 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.65 (t, J = 6 Hz, 2H), 3.00 (s, 3H, SCH_3), 3.02 (t, 2H, J = 6 Hz, SCH_2), 7.01 (m, 1H, ArH), 7.19 (m, 1H, ArH), 7.45 (m, 1H, ArH), 7.93 (m, 1H, ArH); *m/z* (ESI): 302 (MH^+); HRMS (ESI): M^+ calcd for $C_{15}H_{11}NO_2S_2$ 301.0231, found: 301.0238.

4.6. General procedure for the synthesis of 4-sec.amino-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine-3-carbonitriles (**9**)

A mixture of 4-methylthio-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine-3-carbonitrile (**8**, 1 mmol) and *sec*.amine (1.1 mmol) was refluxed in absolute methanol for 7 h. During this period a precipitate separated out which was filtered after cooling. The precipitate was washed with cold ethanol and finally crystallized with acetone.

4.6.1. 10-Methoxy-4-morpholino-2-oxo-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine-3-carbonitrile (**9a**)

Yellow amorphous solid; mp 146 °C; IR (KBr): 2211 (CN) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.57 (t, J = 6.0 Hz, 2H, CH_2), 3.63 (m, 4H, 2 × NCH_2), 3.78 (m, 7H, 2 × OCH_2 & OCH_3), 4.55 (t, J = 6.0 Hz, 2H, OCH_2), 7.14 (m, 3H, Ar–H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.4, 52.0(2C), 55.6, 66.5 (2C), 77.6, 77.9, 112.2 (2C), 112.7, 119.6, 123.5, 125.5, 149.8, 155.2, 158.1, 160.8, 166.0; *m/z* (ESI): 355 (MH^+); HRMS (ESI): MH^+ calcd for $C_{19}H_{19}N_2O_5$ 355.1294, found 355.1289.

4.6.2. 10-Chloro-2-oxo-4-(piperidin-1-yl)-5,6-dihydro-2H-benzo[b]pyrano[2,3-d]oxepine-3-carbonitrile (**9b**)

Buff colored solid; mp 220 °C; IR (KBr): 2217 (CN), 1717 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 1.63 (bs, 6H, 3 × CH_2); 2.65 (t, 2H, J = 5.12 Hz, CH_2); 3.45 (bs, 4H, 2 × NCH_2) 4.51 (t, 2H, J = 5.12 Hz, OCH_2); 7.13 (d, 1H, J = 8.80 Hz, Ar–H); 7.50 (d, 1H, J = 8.80, Ar–H); 7.65 (s, 1H, Ar–H); ^{13}C NMR ($CDCl_3$): δ 18.2, 26.9, 28.2, 41.1, 74.2, 93.1, 114.6, 116.2, 123.5, 124.7, 127.5, 135.9, 136.4, 155.5, 156.3, 158.1, 160.7, 168.0, 200.8; *m/z* (ESI): 356 (M^+); HRMS (ESI): M^+ calcd for $C_{19}H_{17}N_2O_3Cl$ 356.0928, found 356.0928.

4.7. General procedure for the synthesis of 3-amino-4,11,12-trihydro-4-oxo-1H-pyrazolo[4,3-c]benzo[b]oxepino[4,5-e]pyrans (**10**)

A mixture of 4-methylthio-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine-3-carbonitrile (**8**, 1 mmol) or 4-*sec*.amino-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine-3-carbonitrile (**9**, 1 mmol) and hydrazine (98%, 2.1 mmol) in methanol was stirred at room temperature for 18 h. After completion, the reaction mixture was poured onto crushed ice with vigorous stirring and the precipitate obtained was filtered, washed with water and finally purified by crystallization from a mixture of 4% DMF in methanol.

4.7.1. 3-Amino-7-methoxy-4,11,12-trihydro-4-oxo-1H-pyrazolo[4,3-c]benzo[b]oxepino[4,5-e]pyran (**10a**)

Yellow solid; mp 180 °C; IR (KBr): 3406 & 3331 (NH_2 & NH), 1702 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 2.94 (bs, 2H, CH_2), 3.76 (s, 3H, OCH_3), 4.29 (bs, 2H, OCH_2), 6.62 (bh, 2H, NH_2), 6.99 (m, 2H, Ar–H), 7.36 (s, 1H, Ar–H), 12.32 (bh, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.2, 55.5, 71.4, 79.0, 112.3, 114.5, 122.1, 123.4, 151.1, 154.8, 155.9; *m/z* (ESI): 300 (MH^+); HRMS (ESI): MH^+ calcd for $C_{15}H_{14}N_3O_4$ 300.0984, found 300.0974.

4.7.2. 3-Amino-7-chloro-4,11,12-trihydro-4-oxo-1H-pyrazolo[4,3-c]benzo[b]oxepino[4,5-e]pyran (**10b**)

Colorless solid; mp 250 °C; IR (KBr): 3452 & 3370 (NH_2 & NH), 1700 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.01 (t, J = 4.40 Hz, 2H, CH_2), 4.33 (t, J = 4.40, 2H, OCH_2), 6.33 (bh, 2H, NH_2), 7.08 (s, 1H, Ar–H), 7.35 (d, J = 5.84 Hz, 1H, Ar–H), 7.88 (d, J = 5.84 Hz, 1H, Ar–H), 12.25 (s, 1H, NH); *m/z* (ESI): 304 (MH^+); HRMS (ESI): MH^+ calcd for $C_{14}H_{11}ClN_3O_3$ 304.0489, found 304.0498.

4.7.3. 3-Amino-4,11,12-trihydro-4-oxo-1H-pyrazolo[4,3-c]benzo[b]oxepino[4,5-e]pyran (**10c**)

Colorless solid; mp 272 °C; IR (KBr): 3450 (NH_2 & NH), 1702 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 3.00 (t, J = 4.00 Hz, 2H, CH_2), 4.34 (t, J = 4.00, 2H, OCH_2), 6.55 (bh, 2H, NH_2), 7.05 (d, J = 6.60 Hz, 1H, Ar–H), 7.16 (s, 1H, Ar–H), 7.32 (s, 1H, Ar–H), 7.94 (d, J = 7.36 Hz, 1H, Ar–H), 12.25 (s, 1H, NH); *m/z* (ESI): 269 (M^+); HRMS (ESI): M^+ calcd for $C_{14}H_{11}N_3O_3$ 269.0800, found 269.0809.

*4.7.4. 3-Amino-7,8-dimethyl-4,11,12-trihydro-4-oxo-1H-pyrazolo [4,3-c]benzo[b]oxepino[4,5-e]pyran (**10d**)*

Colorless solid; mp 286 °C; IR (KBr): 3538, 3409 (NH_2 & NH), 1698 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 2.38 (s, 6H, 2 × CH_3) 2.98 (t, J = 4.00 Hz, 2H, CH_2), 4.34 (t, J = 4.00, 2H, OCH_2), 6.55 (bh, 2H, NH_2), 7.16 (s, 1H, Ar–H), 7.32 (s, 1H, Ar–H), 12.25 (s, 1H, NH); *m/z* (ESI): 298 (MH^+); HRMS (ESI): MH^+ calcd for $C_{16}H_{16}N_3O_3$ 298.1192, found 298.1201.

*4.7.5. 3-Amino-4,11,12-trihydro-4-oxo-1H-pyrazolo[4,3-c]benzo[b] thiepino[4,5-e]pyran (**10e**)*

Colorless solid; mp 270 °C; IR (KBr): 3439, 3347 (NH_2 & NH), 1692 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 2.64 (bs, 2H, CH_2), 3.52 (bs, 2H, SCH_2), 6.57 (bh, 2H, NH_2), 7.39–7.62 (m, 4H, Ar–H), 12.25 (s, 1H, NH); *m/z* (ESI): 286 (MH^+); HRMS (ESI): MH^+ calcd for $C_{14}H_{12}N_3O_2S$ 286.0650, found 286.0691.

*4.8. General procedure for the synthesis of 3-amino-5,12,12a-trihydro-4-oxo-1H-pyrazolo[4,3-e]thiochromeno[4,3-c][1,2] diazepines (**12**)*

A mixture of 4-methylthio-2-oxo-2,5-dihydrothiochromeno [4,3-*b*]pyran-3-carbonitriles (**3**, 1 mmol) and hydrazine (98%, 4.1 mmol) in ethanol (6 mL) was refluxed for 4 h. Thereafter, the reaction mixture was cooled to room temperature. The precipitate obtained was filtered, washed with methanol and dried in vacuo to give the desired compound (**12**) as a white powder.

*4.8.1. 3-Amino-5,12,12a-trihydro-4-oxo-1H-pyrazolo[4,3-e] thiochromeno[4,3-c][1,2]diazepine (**12a**)*

Yellow solid; IR (KBr): 3428, 3280 (NH_2 & NH), 1657 (C═O) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 4.02–4.22 (m, 3H), 6.04 (bh, 2H), 7.18–7.32 (m, 4H), 7.76 (bh, 1H), 10.30 (bh, 1H); *m/z* (ESI): 286 (MH^+); HRMS (ESI): MH^+ calcd for $C_{13}H_{12}N_5OS$ 286.0718, found 286.0726.

*4.8.2. 3-Amino-8-Chloro-5,12,12a-trihydro-4-oxo-1H-pyrazolo [4,3-e]thiochromeno[4,3-c][1,2] diazepine (**12b**)*

Yellow solid; IR (KBr): 3420, 3282 (NH_2 & NH), 1661 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 4.00–4.24 (m, 3H), 6.09 & 6.56 (bh, 2H), 7.38 (m, 3H), 10.38 (bh, 1H), 11.84 (bh, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.6, 27.8, 123.6, 127.1, 129.0, 129.1, 130.0, 130.4, 130.5, 132.1, 136.0, 157.5, 163.8; *m/z* (ESI): 320 (MH^+); HRMS (ESI): MH^+ calcd for $C_{13}H_{11}ClN_5OS$ 320.0373, found 320.0381.

*4.9. General procedure for the synthesis of 3-amino-5,12,13,13a-tetrahydrobenzo[b]oxepino[4,5-e]pyrazolo[3,4-e][1,2]diazepin-4(1H)-one (**13**)*

A mixture of 4-methylthio-2-oxo-5,6-dihydro-2*H*-benzo[*b*]pyrano[2,3-*d*]oxepine-3-carbonitrile (**9**, 1 mmol) and hydrazine (98%, 4.1 mmol) in DMF (15%) and methanol (6 mL) was refluxed for 4 h. Thereafter, the reaction mixture was cooled to room temperature. The precipitate obtained was filtered, washed with methanol and dried in vacuo to give the desired compound (**13**) as a white powder.

*4.9.1. 3-Amino-8-chloro-5,12,13,13a-tetrahydrobenzo[b]oxepino [4,5-e]pyrazolo[3,4-e][1,2]diazepin-4(1H)-one (**13a**)*

Pale yellow solid; mp 300 °C; IR (KBr): 3582 (NH_2), 3282, 1635 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 2.36 (m, 2H, CH_2), 2.68 (s, 1H, CH), 4.22 (m, 2H, OCH_2), 6.10 (bh, 2H, NH_2), 7.04 (d, J = 7.32 Hz, 1H, Ar–H), 7.24 (d, J = 7.32 Hz, 1H, Ar–H), 7.39 (s, 1H, Ar–H), 10.31 (bh, 1H, CONH), 11.80 (bh, 1H, NH); *m/z* (ESI): 318 (MH^+); HRMS (ESI): MH^+ calcd for $C_{14}H_{13}ClN_5O_2$ 318.0758, found 318.0764.

*4.9.2. 3-Amino-5,12,13,13a-tetrahydrobenzo[b]oxepino[4,5-e] pyrazolo[3,4-e][1,2]diazepin-4(1H)-one (**13b**)*

Pale yellow solid; mp 250 °C; IR (KBr): 3528, 3436 (NH_2 & NH), 1707 (C═O) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 2.38 (m, 2H, CH_2), 2.71 (s, 1H, CH), 4.24 (m, 2H, OCH_2), 6.14 (bh, 2H, NH_2), 7.04 (d, J = 7.30 Hz, 1H, Ar–H), 7.24 (d, J = 7.30 Hz, 1H, Ar–H), 7.39 (m, 2H, Ar–H), 10.31 (bh, 1H, CONH), 11.80 (bh, 1H, NH); *m/z* (ESI): 284 (MH^+); HRMS (ESI): MH^+ calcd for $C_{14}H_{14}N_5O_2$ 284.1147, found 284.1156.

*4.9.3. 4-methylthio-2-oxo-5,6-dihydro-2H-naphtho[b]oxepino[5,4-b]pyran-3-carbonitrile (**15**)*

Powdered KOH (1.5 mmol) was added to a stirred solution of 3,4-dihydronaphtho[1,2-*b*]oxepin-5(2*H*)-one (**14**, 1 mmol) and methyl 2-cyano-3,3-bis(methylthio)acrylate (**1**, 1 mmol) in 8 mL DMF and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into the crushed ice and stirred for 1 h to yield pale-yellow solid. The solid was filtered, dried and crystallized with $CHCl_3$/hexane as pale-yellow needles, mp 148 °C; IR (KBr): 2213 (CN), 1701 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 1.57 (s, 3H, CH_3), 3.03 (t, 2H, J = 5.4 Hz, CH_2), 4.76 (t, 2H, J = 5.4 Hz, OCH_2), 7.59 (m, 3H, Ar–H), 7.83 (d, 1H, J = 7.8 Hz, Ar–H), 7.92 (d, 1H, J = 9.0 Hz, Ar–H), 8.31(d, 1H, J = 8.1 Hz, Ar–H); *m/z* (ESI): 336 (MH^+); HRMS (ESI): MH^+ calcd for $C_{19}H_{14}NO_3S$ 336.0616, found 336.0610.

*4.9.4. 3-Amino-13,14-dihydronaphtho[1,2-b]oxepino[5,4-b] pyrazolo[3,4-e]pyran-4(1H)one (**16**)*

A mixture of **15** (1 mmol) and hydrazine (98%, 2.1 mmol) in methanol (6 mL) was stirred for 18 h. Thereafter, the precipitate obtained was filtered, washed with methanol and dried in vacuo to give the desired compound **16** as a white powder; mp 294 °C; IR (KBr): 3528, 3436 (NH_2 & NH) 1707 (C═O) cm^{-1}; ^1H NMR: (300 MHz, DMSO-d_6): δ 3.09 (t, 2H, J = 5.4 Hz, CH_2), 4.23 (t, 2H, J = 5.4 Hz, OCH_2), 6.26 (bh, 2H, NH_2), 7.57 (m, 2H, Ar–H), 7.70 (d, 1H, J = 9.0 Hz, Ar–H), 7.90 (m, 1H, Ar–H), 8.01 (d, 1H, J = 9.0 Hz, Ar–H), 8.26 (m, 1H, Ar–H), 13.96 (bh, 1H, NH); ^{13}C NMR: (100 MHz, DMSO-d_6): δ 27.4, 72.5, 117.8, 122.2, 122.8, 124.5, 126.3, 126.9, 127.3, 127.5, 134.0, 147.9, 151.5, 153.4, 158.0; *m/z* (ESI): 320 (MH^+); HRMS (ESI): MH^+ calcd for $C_{18}H_{14}N_3O_3$ 320.1035, found 320.1044.

*4.10. Crystal data for 3-amino-4,11,12-trihydro-4-oxo-1H-pyrazolo [4,3-c]benzo[b]thiepino[4,5-e]pyran (**10e**)*

Crystals of X-ray quality for **10e** were obtained by slow evaporation of the solution of compound in 1:1 DMF:H_2O mixture at room temperature, Molecular formula $C_{14}H_{13}N_3O_3S$, formula mass 303.33, monoclinic space group $P2_1/n$, a = 10.4061(9), b = 9.1976(7), c = 13.5965(12) Å, β = 91.494(6)°, V = 1300.89(19)Å^3, Z = 4, d_{calcd} = 1.549 mg m^{-3}, linear absorption coefficient 0.264 mm^{-1}, $F(000)$ = 632, crystal size 0.21 × 0.20 × 0.13 mm, reflections collected 14,038, independent reflections 3130, Final indices [$I > 2\sigma(I)$] R_1 = 0.0358 wR_2 = 0.0838, R indices (all data) R_1 = 0.0565, wR_2 = 0.0924, gof 1.020, Largest difference peak and hole 0.327 and −0.262 eÅ^{-3}.

4.11. Cytotoxic evaluation of synthesized compounds on carcinoma cells Colo-205, HepG2 and normal cells IEC-6

4.11.1. Cell culture

The human colorectal carcinoma cells (Colo-205), human hepatocarcinoma cells (HepG2) and rat normal intestinal epithelial

cells (IEC-6) were procured from cell repository, National Centre for Cell Sciences (NCCS), Pune. Colo-205 cells, HepG2 cells and IEC-6 cells were cultured in RPMI-1640, Eagles Minimum Essential Medium (EMEM) and Dulbecco's modified Eagle's medium (DMEM), respectively supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid cell culture incubator (Eppendorf).

4.11.2. Cell viability assay

The effect of **5a**,**b**; **10a**,**c**,**d**; **12b**; **13b**; **16** and 1-methylthio-5,6-dihydrobenzo[*f*]quinolin-3-amine [18] (see Supplementary data) on the viability of Colo-205, HepG2 and IEC-6 cells were determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) assay [19]. In brief, IEC-6, Colo-205 and HepG2 cells (4×10^3–5×10^3) were treated with various doses of synthetic compounds (0–100 μM) for 24 and 48 h in 96 well plates. A 10 μL MTT solution (5 mg/mL PBS) was added to the wells and the plates were further incubated for 4 h at 37 °C in a humidified CO_2 incubator. On completion, the plates were centrifuged at 1200 rpm for 10 min, media was discarded and 200 μL DMSO was added to each well to dissolve formazan formed and after shaking for 20 s, the plates were read at 550 nm on a multi plate reader (Bio-tek, Winooski, VT). The effect of the synthetic compounds on cell viability was assessed as the percentage of cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability as shown in Fig. 4. The data are shown as the percentage of cell viability and represent mean ± standard errors of three experiments.

4.11.3. IC_{50} calculation

IC_{50} values were calculated by plotting the graph between concentration (μM) of synthesized compound at *X*-axis and the cell viability data on *Y*-axis and fitted the data with a straight line with 50% cell death (linear regression). IC_{50} value is then estimated using the fitted line by drawing a perpendicular on *X*-axis.

4.11.4. Analysis of apoptotic cell death

Apoptosis was detected in Colo-205 and HepG2 cells using Annexin V-FITC kit through flow cytometer according to the manufacturer's protocol (BD Biosciences, San Jose, CA) as described earlier [20]. Briefly, Colo-205 cells (5×105) were treated with **10a**, **10d** and **16** compounds for 24 and 48 h, while HepG2 cells (5×105) were treated with **10c** and **13b** compounds for 48 h at IC_{50} concentrations. The harvested cells (Colo-205 or HepG2) were suspended in 1 mL binding buffer (1×) supplied with the reagent kit (BD Biosciences, San Jose, CA). An aliquot of 100 μL was incubated with 5 μL Annexin-V-FITC and 5 μL PI for 15 min in dark at room temperature and 400 μL binding buffer (1×) was added to each sample. The FITC and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively and 10,000 events were acquired on flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

4.11.5. Western blot analysis of proteins involved in apoptotic machinery

For western blot analysis of various proteins, 60 μg of protein lysate was resolved on 10% SDS-polyacrylamide gel and the proteins were transferred to PVDF membranes [20]. The blotted membrane was blocked with either 5% non-fat dry milk or 5% BSA in PBS containing 0.1% Tween 20 (blocking solution), and incubated with specific antibodies against p53, p21/waf1, Bax, Bcl-2, cytochrome *c*, caspase 3, 8, 9 and PARP at dilutions indicated by the manufacturer. The blots were further incubated with HRP-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO) and developed by ECL Western Blotting Detection Kit as described in the manufacturer's protocol (Amersham, Fairfield, CT). All the blots were stripped and reprobed with β-actin to ensure equal loading of protein.

4.11.6. Statistical analysis

The results were expressed as the mean ± standard error (SE) in Figs. 4 and 6. The statistical significance of difference between the values of control and treatment groups was determined using Student's *t*-test. A *P* value of <0.05 was considered statistically significant. For IC_{50} experiments (Figs. 5 and 7) mean of duplicate readings are plotted in the graph.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.013.

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