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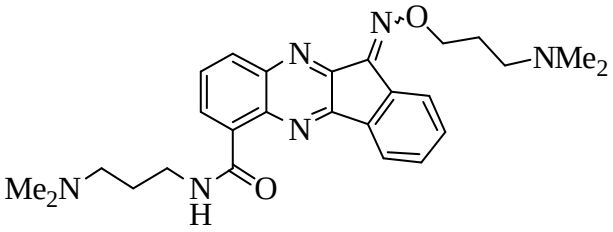


**10a**

$IC_{50}$ = 0.87 μM (MDA-MB231)
0.82 μM (PC-3)
0.64 μM (Huh-7)
> 10 μM (MRC-5)

Compound **10a** induced cell cycle arrest at S phase *via* activation of caspase-3, -7 and increase in the expression of Bad and Bax but decrease in Bcl-2 and PARP.

# Discovery of Indeno[1,2-*b*]quinoxaline Derivatives as Potential Anticancer Agents

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**Abstract**

We have synthesized certain indeno[1,2-*b*]quinoxaline derivatives for antiproliferative evaluation. Among them, 11-{[3-(dimethylamino)propoxy]imino}-*N*-[3-(dimethylamino) propyl]-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide (**10a**) was active against the growth of MDA-MB231, PC-3, and Huh-7 with $IC_{50}$ values of 0.87 (selectivity index, SI = 36.22), 0.82 (SI = 38.43), and 0.64 μM (SI = 49.23) respectively. Compound **10a** was inactive against the growth of normal human fetal lung fibroblast cell line (MRC-5) with an $IC_{50}$ value of 31.51 μM. Its analogs, **10b** and **10c**, were also active against the growth of MB231, PC-3, and Huh-7 with $IC_{50}$ values of < 1.0 μM in each case. Our results have also indicated compounds **10a** – **10c** exhibited comparable inhibitory activities against topo I and topo II with the positive compound **2** at a concentration of 10 μM. Mechanism studies indicated that compound **10a** induced cell cycle arrest at S phase *via* activation of caspase-3, -7 and an increase in the protein expression of Bad and Bax but a decrease in expression of Bcl-2 and PARP, which consequently cause cell death. In addition, compound **10a** attenuated the levels of phosphorylated Src, Akt-1, and Akt-2 protein levels but did not affect the total protein expression of Akt. We have also implanted human hepatocellular carcinoma cells into the yolk sac of zebrafish larvae and incubated larvae with various concentrations of **10a**. Our results of the zebrafish xenograft assay confirmed the anti-tumor effect of **10a** *in vivo*.

Key words: Indeno[1,2-*b*]quinoxaline derivatives; Zebrafish xenograft assay, Anticancer agents; Cell cycle; Apoptosis; Antiproliferative activity.

## 1. Introduction

A number of polycyclic nitrogen heterocycles, for example, ellipticine [1-3], batracylin [4,5], and anthracycline antibiotics are of interest as anticancer agents whose action is based mainly on DNA damage such as DNA intercalation and the inhibition of topoisomerase II. Ellipticine (5, 11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole) is a potent antitumor alkaloid bearing a tetracyclic ring system and was first isolated from the evergreen tree *Ochrosia elliptica* Labill. Batracylin (8-aminoisoindolo[1,2-*b*]quinazolin-12(10*H*)-one) is a heterocyclic amine that exhibits antitumor activity in a number of *in vivo* and *in vitro* models. However, the most noticeable examples of DNA-intercalating agents are anthracycline antibiotics such as doxorubicin and daunorubicin which possess a tetracyclic system where three of the rings are coplanar.

The versatile quinoxaline ring constitutes a large number of potential bioactive agents which possess broad spectrum of pharmacological activities such as anti-infective [6-8], anti-inflammatory [9,10], and anticancer activities [11-13]. Recently, we have synthesized and evaluated antiproliferative activities of certain DNA intercalating agents with the tetracyclic indeno[1,2-*c*]quinoline scaffold [14-20]. Among them, 9-methoxy-6-(piperazin-1-yl)-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-3-aminopropyl oxime (**1**) and *(E)*-6-hydroxy-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-2-(pyrrolidin-1-yl)ethyl oxime (**2**) exhibited $IC_{50}$ value of 0.64 and 0.89 μM respectively against the growth A549, which was more active than camptothecin and topotecan. 9-Methoxy-6-{4-[(oxiran-2-yl) methyl]piperazin-1-yl}-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-(oxiran-2-yl)methyl oxime (**3**) was also found to exhibit strong antiproliferative activities aginst the growth of Hela, SKHep, MDA-MB-231, and H1299 cells with an $IC_{50}$ value of 0.54, 0.99, 0.79, and 1.18 μM respectively [20]. In continuation of our search for potentential anticancer agents, the present study describes the preparation and antiproliferative activities of certain tetracyclic indenoquinoxaline derivatives (*Figure* 1) whose structures can be considered as aza analogs of indenoquinoline derivatives.

< **Insert *Figure* 1 here** >

## 2. Chemistry

Treatment of the known 11*H*-Indeno[1,2-*b*]quinoxalin-11-one (**4a**) [21] with $NH_2OH$ gave 11*H*-Indeno[1,2-*b*]quinoxalin-11-one oxime (**5a**) in 94% yield. Its aminoalkyl derivatives **6a**, **7a** – **7c** were obtained as hydrochloride salts by the reaction of **4a** with various aminoalkoxyamines followed by the treatment of 3N HCL as described in *Scheme* 1. Preparation of 6-carboxamide derivatives is outlined in *Scheme* 2. Reaction of 11*H*-Indeno[1,2-*b*]quinoxalin-11-one-6-carboxylic acid (**4c**) [21] and 1,1'-carbonyldiimidazole gave the imidazole intermediate **8** which was treated with various amines to afford C-6 amide derivatives **9a** - **9d**. Treatment of compounds **9a** - **9d** with various aminoalkoxyamine gave C-11 aminoalkoxyimino derivatives **10a** - **10d** in good overall yields.

< **Insert *Scheme 1 and 2* here** >

The preparation of di-aminoalkoxyimino derivatives **14** and **15** are described in *Scheme 3*. Reaction of **4b** with NBS gave 6-(bromomethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one (**11**) which was then oxidized with $MnO_2$ to give 11-oxo-11*H*-indeno[1,2-*b*]quinoxaline-6-carbaldehyde (**12**) in a high overall yield. Treatment of **12** with $NH_2OH$ or aminoalkoxyamines gave di-hydroxyimine **13** or di-aminoalkoxyimino derivatives **14** and **15** respectively. Compound **11** was also treated with various secondary amines to give C-6 aminomethyl substituted intermediates **16a** – **16e** respectively which was converted to C-11 aminoalkoxy derivatives **17a** – **17e** in fairly good overall yields as shown in *Scheme* 4.

< **Insert *Scheme 3 and 4* here** >

## 3. Results and Discussion.

### 3.1. Antiproliferative Activity and DNA Unwinding.

All the newly synthesized compounds were evaluated *in vitro* against four cancer cell lines (MDA-MB231, H1299, PC-3 and Huh-7) using XTT assay [22]. The normal human fetal lung fibroblast cell line (MRC-5) was also evaluated since a potential anticancer drug

candidate should selectively affect only tumor cells and not somatic cells. The concentration that inhibited the growth of 50% of cells ($IC_{50}$) was determined from the linear portion of the curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells, by 50%. The results of $IC_{50}$ values are summarized in *Table* 1. Indeno[1,2-*b*]quinoxalin-11-one (**4a**) and its 11-hydroxyimino derivative **5a** were inactive against the growth of all cell lines tested. However, introduction of the aminoalkyl side chain at C-11 position improved growth inhibitory effect in which compounds **6a** and **7a** exhibited moderate antiproliferative activity. Further substitution of methyl group at C-5, compound **4b**, did not improve growth inhibitory effect. Indeno[1,2-*b*]quinoxaline-11-oxo-5-carboxylic acid (**4c**) was also inactive against the growth of all cell lines tested. Its C-5 amide derivatives exhibited moderate effect against certain types of tested cells with exception of compounds **9d** which is inactive. Further introduction of aminoalkoxyimino side chains on C-11 position of these amide derivatives enhanced antiproliferative activity. Among them, 11-{[3-(dimethylamino)propoxy]imino}-*N*-[3-(dimethylamino)propyl]-11*H*-indeno[1,2-*b*]-quinoxaline-6-carboxamide (**10a**) was active against the growth of MDA-MB231, PC-3, and Huh-7 with $IC_{50}$ values of 0.87 (selectivity index, SI = 36.22), 0.82 (SI = 38.43), and 0.64 μM (SI = 49.23) respectively. Compound **10a** was inactive against the growth of normal human fetal lung fibroblast cell line (MRC-5) with an $IC_{50}$ value of 31.51 μM. Its analogs, **10b** and **10c**, were also active against the growth of MDA-MB231, PC-3, and Huh-7 with $IC_{50}$ values of < 1.0 μM in each case. Compound **10d** was selectively active against the growth of MDA-MB231 with an $IC_{50}$ value of < 1.0 μM and was found to be less active against the growth of normal human fetal lung fibroblast cell line (MRC-5) with $IC_{50}$ values of 11.52 μM. Compound **12** was inactive while its dihydroxyimino derivative **13** was marginally active against all cell lines tested. Antiproliferative activities were further enhanced by the introduction of aminoalkyl side chains. The same structure-activity relationship (SAR) was observed for compounds **16** and **17** in which C-11 oxo derivatives **16a - 16e** were inactive while their aminoalkoxyimino counterparts **17a – 17c** exhibited

potent inhibitory activities on all the cells tested. However, **17d** was only marginally active while **17e** was selectively active against MDA-MB231 and Huh-7 with $IC_{50}$ values of < 1.0 μM. Compound **10a** was found to inhibit the growth of MDA-MB231, PC-3 and Huh-7 but not MRC-5 and therefore was selected for further mechanism studies.

Previous studies [16-20] indicated that most of the indeno[1,2-*c*]quinolin-11-one derivatives interact with DNA and therefore, DNA unwinding assay of these newly synthesized indeno[1,2-*b*]quinoxaline derivatives was carried out. Results from *Table* 1 indicated that compounds **10a-d**, **14** and **17a-c** are capable of intercalating DNA.

< **Insert *Table 1* here** >

### 3.2. Topoisomerase Inhibition.

The topoisomerase I (topo I) and topoisomerase II (topo II) inhibitory activities of these indeno[1,2-*b*]quinoxaline derivatives were carried out with the known compound **2** as a positive control [16]. Results are summarized in *Fig*ure 2 and Table 2. All compounds were evaluated to inhibit topo I at 100 μM (*Figure* 2A) and 10 μM (*Figure* 2B) respectively. From the gels shown in *Figure* 2A, most of them exhibited moderate inhibition of Topo I at 100 μM. However, only compounds **10a** – **10d**, **14**, **15**, **17a** – **17c**, and **17e** exhibited moderate inhibition of Topo I at 10 μM. Quantitation of Topo II inhibition was also analyzed at 100 μM (*Figure* 2C) and 10 μM (*Figure* 2D) respectively. Compounds **6a**, **7a**, **7b**, **9a** – **9c** demonstrated strong topo II inhibitory activities at 100 μM (*Figure* 2C) while compounds **10a** – **10d**, **14**, and **15** inhibit topo II with moderate activities. However, only compounds **10a** – **10c**, **14**, and **15** exhibited moderate inhibition of Topo II at 10 μM (*Figure* 2D).

Our results indicated compounds **10a** – **10c**, **14**, and **15** exhibited comparable inhibitory activities against topo I and topo II with the positive compound **2** at a concentration of 10 μM. These compounds could be further optimized to discover dual topo I/II inhibitory agents.

< **Insert *Figure 2* and *Table 2* here** >

### 3.3. Compound 10a induces growth inhibition and S-phase arrest in Huh-7 cells.

To investigate the mechanisms leading to loss of cell proliferation by **10a**, the effects of

**10a** treatment on cell cycle arrest or apoptosis were examined. As shown in *Fig*ure 3 and *Table* 3, cells treated with 1-10 μM of **10a** for 24h notably increased the percentage of cells in the S-phase from 11.4% to 38.4%, with a parallel reduction in the percentage of cells in G0/G1 and G2/M phase, while the hypodiploid (Sub G1 phase) cells increased. These results suggested that **10a** inhibited the cellular proliferation of Huh-7 cells *via* S-phase arrest of the cell cycle. Morphological changes of cells treated with **10a** can be visually observed with light microscopy (*Figure* 4). We found that the Huh-7 cells treated with **10a** at 5 μM for 24 h became shrinked. Such morphological changes were not apparent in the control cells.

< **Insert *Figure 3, Table 3, and Figure 4* here** >

### 3.4. Compound 10a causes a decrease in the expression levels of cyclins D1, A, and B1.

Perturbation in cell cycle progression mediated by alterations in cell cycle-related proteins plays a vital role in the proliferation of cancer cells. To elucidate the role of these molecules in the inhibition of cell cycle induced by **10a** treatment, protein extract was prepared from the **10a**-treated cells for 24 h. As shown in *Figure* 5, **10a** treatment of 1-10 μM caused a marked decrease in cyclin A, cyclin B1, and cyclin D1 expression. These results suggested that **10a** induced S-phase arrest, possibly by altering the S-phase cell cycle-related protein expression in Huh-7 cells.

< **Insert *Figure 5* here** >

### 3.5. Compound 10a-induced apoptosis in Huh-7 cells

To understand the mechanism of **10a**-induced apoptosis, we examined the changes of the intracellular proteins related to apoptosis, such as Bcl-2 family proteins (Bcl-2 and Bax), caspase-3, -7, and PARP in cells treated with **10a**. Bcl-2 is the first identified member of a large family of apoptosis-regulating proteins, consisting of blockers (such as Bcl-2) and promoters (such as Bax) of cell death. Caspases play a crucial role in apoptotic cell death. Caspase-3 and -7 in particular, could be activated by the proteolytic processing of procaspase 3 and -7 in response to exogenous apoptosis inducers [23]. PARP is involved in DNA repair predominantly in response to environmental stress, and is important for the maintenance of

cell viability [24]. Our results indicated that caspase-3 and -7 were activated and increased the protein expression of Bax, but decreased expression of Bcl-2 and PARP after the treatment of **10a** for 24 h in a concentration-dependent manner (*Fig*ure 6). Thus, compound **10a** induced cell cycle arrest at S phase *via* activation of caspase-3, -7 and an increase in the protein expression of Bad and Bax but a decrease in expression of Bcl-2 and PARP, which consequently cause cell death.

< **Insert Figure 6 here** >

### 3.6. Compound 10a attenuates activation of Src and Akt signaling

The signaling pathways include the Src and Akt, which play a significant role in mitogenic and cell survival [25]. Akt/protein kinase B (PKB) is the downstream molecules of PI3K and previous studies have demonstrated that Akt plays a major role in the survival of cells under a variety of conditions [26]. To examine whether the activities of Src and Akt are down-regulated by **10a**, the phosphorylation of Src, Akt-1 and Akt-2 activation in Huh-7 cells was analyzed. As shown in *Figure* 7, Compound **10a** treatment attenuated the levels of phosphorylated Src, Akt-1 and Akt-2 protein levels of in **10a**-treated cells. However, **10a** did not affect the total protein expression of Akt.

< **Insert *Figure 7* here** >

### 3.7. Antitumor efficacy of 10a in zebrafish xenograft assay

To examine the anti-tumor effect of **10a** *in vivo*, we implanted human hepatocellular carcinoma cells into the yolk sac of zebrafish larvae and incubated larvae with indicated concentrations of **10a**. The intensity of red fluorescence is proportional to the xenograft tumor size. Results indicated compound **10a** significantly reduced tumor size in a dose-dependent manner as shown in *Figure* 8.

< **Insert *Figure 8* here** >

## 4. Conclusion

A number of indeno[1,2-*b*]quinoxaline derivatives were synthesized and evaluated for antiproliferative activities. Among them, 11-{[3-(dimethylamino)propoxy]imino}-*N*-[3-

(dimethylamino)propyl]-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide (**10a**) was active against the growth of MB231, PC-3, and Huh-7 with $IC_{50}$ values of 0.87, 0.82, and 0.64 μM respectively. Compound **10a** was inactive against the growth of MRC-5 with an $IC_{50}$ value of 31.51 μM. Our results have also indicated compound **10a** exhibited comparable inhibitory activities against topo I and topo II with the positive compound **2** at a concentration of 10 μM. Mechanism studies indicated that compound **10a** induced cell cycle arrest at S phase *via* activation of caspase-3, -7 and an increase in the protein expression of Bad and Bax but a decrease in expression of Bcl-2 and PARP, which consequently cause cell death. We have also implanted human hepatocellular carcinoma cells into the yolk sac of zebrafish larvae and incubated larvae with various concentrations of **10a**. Our results of the zebrafish xenograft assay confirmed the anti-tumor effect of **10a** *in vivo*. Further structural optimization of compound **10a** is on-going.

## 5. Experimental

### 5.1. General.

TLC: precoated (0.2 mm) silica gel 60 $F_{254}$ plates from EM Laboratories, Inc.; detection by UV light (254 nm). All chromatographic separations were performed using silica gel (Merck 60 230–400 mesh). M.p.: Yamato MP-21 melting-point apparatus; uncorrected. $^{1}H$ and $^{13}C$ NMR spectra: Varian-Unity-400 spectrometer at 400 and 100 MHz, chemical shifts in ppm with $SiMe_4$ as an internal standard (= 0 ppm), coupling constants *J* in Hz. Mass spectra were recorded on Bruker APEX II (ESI) mass spectrometer. Elemental analyses were carried out on a Heraeus CHN-O-Rapid elemental analyzer, and results were within ± 0.4% of calculated values. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC).

### 5.2.1. 11*H*-Indeno[1,2-*b*]quinoxalin-11-one (4a)

A mixture of ninhydrin (1.80 g, 10.0 mmol) and 1,2-phenylenediamine (1.14 g, 10.5 mmol) in EtOH (100 mL) was refluxed for 2 h (TLC monitoring). The mixture was cooled, then the resulting precipitate was filtered, purified by column chromatography ($CH_2Cl_2$), and

crystallized from EtOH/$CH_2Cl_2$ (5/1) to give **4a** (2.16 g, 93% yield) as a yellow solid. Mp: 218-220°C. (lit. mp. 217-219 °C) [21]

**5.2.2. 6-Methyl-11*H*-indeno[1,2-*b*]quinoxalin-11-one (4b).**

Compound **4b** was obtained from ninhydrin (1.80 g, 10.0 mmol), 2,3-diaminotoluene (1.22 g, 10.0 mmol) and EtOH (100 mL) as described for the preparation of **2a** in 85% yield as a yellow needle solid. Mp: 227-229°C. (from EtOH), (lit. mp. 222-224 °C from acetone) [21]

**5.2.3. 11-Oxo-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxylic acid (4c).**

A mixture of ninhydrin (1.80 g, 10.1 mmol) and 2,3-diaminobenzoic acid (1.52 g, 10.0 mmol) in EtOH (100 mL) was refluxed for 2 h (TLC monitoring). The mixture was cooled, then the resulting precipitate was filtered and washed with EtOH (50 mL) to give **2c** (2.65 g, 96% yield) as a yellow solid. Mp: 368-370°C. (decomp.), (lit. mp. > 300 °C from ethylene glycol) [21]

**5.3.1. 11*H*-Indeno[1,2-*b*]quinoxalin-11-one oxime (5a).**

A mixture of **4a** (0.23 g, 1.0 mmol) and hydroxylamine hydrochloride (0.35 g, 5.0 mmol) in EtOH (5.0 mL) was refluxed for 6 h (TLC monitoring). The mixture was cooled and poured into water. The resulting precipitate was filtered and crystallized from EtOH to give **5a** (0.23 g, 94% yield) as a white solid. Mp: 274-276 °C (from EtOH, decomp.). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.67-7.72 (m, 2H, ArH), 7.78-7.85 (m, 2H, ArH), 8.10-8.13 (m, 2H, ArH), 8.14-8.17 (m, 1H, ArH), 8.50-8.53 (m, 1H, ArH), 13.48 (s, 1H, NOH). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 122.26, 128.76, 129.30, 129.87, 129.99, 130.58, 132.09, 132.56, 133.07, 135.99, 141.56, 141.85, 147.18, 150.81, 152.89. ESIMS $[M+H]^+$:248.01. Anal. calc. for $C_{15}H_9N_3O \cdot 0.2\ H_2O$: C 71.81, H 3.78, N 16.75; found: C 71.47, H 3.65, N 16.80. [21]

**5.3.2. 11*H*-Indeno[1,2-*b*]quinoxalin-11-one *O*-[2-(pyrrolidin-1-yl)ethyl] oxime hydrochloride (6a).**

A mixture of **4a** (0.23 g, 1.0 mmol) and 2-(pyrrolidino)ethoxyamine hydrochloride (0.61 g) in EtOH (5.0 mL) was refluxed for 6 h (TLC monitoring). The mixture was cooled and concentrated, then dissolved in water (20 mL), neutralized by $K_2CO_3$ (0.41 g, 3.0 mmol), and

extracted with $CH_2Cl_2$ three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH 10/1). The oil compound was stirred with 3N HCl (3.0 mL) in EtOH (2.0 mL) for 0.5 h, then evaporated in vecuo. The residue was dissolved in EtOH (2.0 mL), then poured into acetone (50 mL). The resulting precipitate was filtered and dried to give **6a** (0.33 g, 87% yield) as a yellow solid. Mp: 187-189 $^{o}$C (decomp.). $^{1}$H NMR (400 MHz, $D_2O$): δ 1.87-1.92 (m, 2H, pyrrolidinyl-H), 2.00-2.05 (m, 2H, pyrrolidinyl-H), 2.96-3.03 (m, 2H, pyrrolidinyl-H), 3.42 (br, 2H, $OCH_2\underline{CH_2}N$), 3.52-3.57 (m, 2H, pyrrolindyl-H), 4.22 (br, 2H, $O\underline{CH_2}CH_2N$), 6.56-6.57 (m, 1H, ArH), 6.74-6.76 (m, 3H, ArH), 6.90-6.93 (m, 1H, ArH), 7.06-7.09 (m, 3H, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 22.78(2C), 53.71, 55.08 (2C), 71.26, 121.10, 127.32, 128.29 (2C), 130.30, 130.48, 131.19, 132.46 (2C), 133.96, 139.22, 139.76, 146.50, 147.53, 150.54. ESIMS $[M+H]^+$: 333.11. Anal. calc. for $C_{21}H_{20}N_4O \cdot 1.0\ HCl \cdot 1.9\ H_2O$: C 60.74, H 6.03, N 13.50; found: C 60.53, H 5.68, N 13.43.

**5.3.3. 11*H*-Indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethylamino)propyl] oxime hydrochloride (7a)**.

A mixture of **4a** (0.23 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.61 g) in EtOH (5.0 mL) was refluxed for 6 h (TLC monitoring). The mixture was cooled and concentrated, then dissolved in water (20 mL), neutralized by $K_2CO_3$ (0.41 g, 3.0 mmol), and extracted with $CH_2Cl_2$ three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH 10/1). The oil compound was stirred with 3N HCl (3.0 mL) in EtOH (2.0 mL) for 0.5 h, then evaporated in vecuo. The residue crystallized from EtOH to give **7a** (0.30 g, 82% yield) as a white solid. Mp: 220-222 $^{o}$C (from EtOH, decomp.). $^{1}$H NMR (400 MHz, $D_2O$): 1.85-1.92 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.81 (s, 6H, $N(\underline{CH_3})_2$), 3.01-3.05 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 3.87 (br, 2H, $O\underline{CH_2}CH_2CH_2N$), 6.61 (d, 1H, $J$ = 6.8 Hz, ArH), 6.67-6.76 (m, 2H, ArH), 6.82 (d, 1H, $J$ = 6.8 Hz, ArH), 6.86-6.88 (m, 1H, ArH), 7.03-7.05 (m, 1H, ArH), 7.15-7.17 (m, 2H, ArH). $^{13}$C NMR (100 MHz, $D_2O$): 23.83, 42.91, (2C), 54.92, 72.66, 121.07, 127.40, 127.82, 128.47, 130.08, 130.54, 130.89, 132.01, 132.20, 133.73, 139.52, 139.77, 145.28, 147.69, 150.44. ESIMS $[M+H]^+$: 345.09. Anal. calc. for

$C_{20}H_{20}N_4O \cdot 1.0\ HCl \cdot 1.2\ H_2O$: C 61.50, H 6.05, N 14.35; found: C 61.17, H 5.92, N 14.29.

**5.3.4. 6-Methyl-11*H*-indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethylamino)propyl] oxime hydrochloride (7b)**.

Compound **7b** was obtained from **4b** (0.25 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) as described for the preparation of **7a** in 85% yield as a white solid. Mp: 231-233 $^{o}$C (from EtOH, decomp.). $^{1}$H NMR (400 MHz, $D_2O$): δ 1.74 (s, 3H, Ar$\underline{CH_3}$), 1.95-2.02 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.81 (s, 6H, $N(\underline{CH_3})_2$), 3.06-3.10 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 4.02 (t, 2H, *J* = 5.6 Hz, $O\underline{CH_2}CH_2CH_2N$), 6.67-6.69 (m, 1H, ArH), 6.76-6.86 (m, 5H, ArH), 7.05 (d, 1H, *J* = 6.8 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 17.03, 24.01, 42.89 (2C), 55.03, 72.58, 121.16, 126.23, 127.93, 129.38, 130.66, 130.97, 131.65, 131.79, 134.75, 136.06, 139.36 (2C), 145.92, 147.14, 150.15. ESIMS $[M+H]^+$: 347.11. Anal. calc. for $C_{21}H_{22}N_4O \cdot 1.0\ HCl \cdot 1.0\ H_2O$: C 62.90, H 6.30, N 13.98; found: C 62.63, H 6.28, N 13.92.

**5.3.5. 11-{[3-(Dimethylamino)propoxy]imino}-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxylic acid hydrochloride (7c).**

Compound **7c** was obtained from **4c** (0.28 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) as described for the preparation of **7a** in 75% yield as a green solid. Mp: 250-252 $^{o}$C (from EtOH, decomp.). $^{1}$H NMR (400 MHz, $D_2O$): δ 2.03-2.10 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.86 (s, 6H, $N(\underline{CH_3})_2$), 3.15-3.19 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 4.12-4.15 (m, 2H, $O\underline{CH_2}CH_2CH_2N$), 6.59 (d, 1H, *J* = 6.8 Hz, ArH), 6.86 (t, 1H, *J* = 6.8 Hz, ArH), 6.92 (t, 1H, *J* = 6.8 Hz, ArH), 7.10 (d, 1H, *J* = 6.8 Hz, ArH), 7.24-7.32 (m, 2H, ArH), 7.79 (dd, 1H, *J* = 6.8, 1.6 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 23.94, 42.92 (2C), 54.79, 73.75, 121.91, 122.47, 128.15, 130.59, 130.98, 131.03, 133.03, 134.61, 134.88, 135.73, 136.92, 139.04, 144.22, 148.52, 148.81, 166.97. ESIMS $[M+H]^+$: 377.12. Anal. calc. for $C_{21}H_{20}N_4O_3 \cdot 1.0\ HCl$: C 61.08, H 5.14, N 13.57; found: C 61.06, H 5.29, N 13.41.

**5.4. General procedure for preparation of compounds 9a-9d.**

A mixture of **4c** (0.55 g, 2.0 mmol) and 1,1'-carbonyldiimidazole (0.97 g, 6.0 mmol) in 1,4-dioxane (20 mL) was refluxed for 4 h (TLC monitoring). The mixture was cooled, concentrated**,** and triturated with water. The resulting precipitate was collected and dried to give intermediate compound **8** (639.8 mg). The compound **8** was stirred in $CH_2Cl_2$ (20 mL) at room temperature, then substituted amines (6.0 mmol) was added, and the reaction mixture was stirred for 10 min (TLC monitoring). The mixture was extracted with water three times, then organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH 10/1) and crystallized from EtOH to give **9a-9d**.

**5.4.1. *N*-[3-(Dimethylamino)propyl]-11-oxo-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide hydrochloride (9a).**

yellow solid (0.45g, 63% yield); Mp: 172-174 $^{o}$C (from EtOH). $^{1}$H NMR (400 MHz, $CDCl_3$): δ 1.97 (quin, 2H, *J* = 6.8 Hz, $NHCH_2\underline{CH_2}CH_2N$), 2.31 (s, 6H, $N(\underline{CH_3})_2$), 2.53 (t, 2H, *J* = 6.8 Hz, $NHCH_2CH_2\underline{CH_2}N$), 3.70 (t, 2H, *J* = 6.8 Hz, $NH\underline{CH_2}CH_2CH_2N$). 7.67-7.71 (m, 1H, ArH), 7.82-7.87 (m, 2H, ArH), 7.96-7.99 (m, 1H, ArH), 8.06 (d, 1H, *J* = 7.6 Hz, ArH), 8.36-8.38 (m, 1H, ArH), 8.89-8.91 (m, 1H, ArH), 10.37 (br, 1H, $CO\underline{NH}$). $^{13}$C-NMR (100 MHz, $CDCl_3$): δ 27.74, 38.18, 45.50 (2C), 57.45, 122.60, 125.12, 130.05, 130.12, 133.42, 135.29, 136.53, 137.00, 137.03, 140.06, 140.38, 142.67, 148.93, 155.31, 164.49, 188.98. ESIMS $[M+H]^+$: 361.10. Anal. calc. for $C_{26}H_{32}N_6O_2 \cdot 2.0\ HCl \cdot 3.0\ H_2O$: C 53.13, H 6.87, N 14.30; found: C 52.90, H 6.89, N 14.22.

**5.4.2. *N*-[2-(Dimethylamino)ethyl]-11-oxo-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide (9b).** yellow solid (0.51 g, 73% yield); Mp: 222-223 $^{o}$C (from EtOH, decomp.), (lit. mp. 220-221 $^{o}$C from EtOH) [27]. ESIMS $[M+H]^+$: 347.05.

**5.4.3. 11-Oxo-*N*-[2-(pyrrolidin-1-yl)ethyl]-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide (9c).** green solid (0.46 g, 62% yield); Mp: 197-199 $^{o}$C (from EtOH, decomp.). $^{1}$H NMR (400 MHz, $CDCl_3$): δ 1.88 (quin, 4H, *J* = 7.6 Hz, pyrrolidinyl-H), 2.74 (br, 4H, pyrrolidinyl-H), 2.88 (t, 2H, *J* = 5.6 Hz, $NHCH_2\underline{CH_2}N$), 3.80 (q, 2H, *J* = 5.2 Hz, $NH\underline{CH_2}CH_2N$), 7.64 (td, 1H, *J* = 1.2, 7.6 Hz, ArH), 7.76 (td, 1H, *J* = 1.2, 7.6 Hz, ArH), 7.84 (t, 1H, *J* = 7.6 Hz, ArH), 7.95

(d, 1H, *J* = 7.6 Hz, ArH), 8.22 (d, 1H, *J* = 7.2 Hz, ArH), 8.35 (dd, 1H, *J* = 7.6, 1.6 Hz, ArH), 8.92 (d, 1H, *J* = 1.6, 7.6 Hz, ArH), 10.63 (br, 1H, CONH). $^{13}$C-NMR (100 MHz, $CDCl_3$): δ 23.61 (2C), 39.18, 54.26 (2C), 55.34, 122.91, 125.00, 129.94, 130.03, 133.29, 135.24, 136.53, 136.62, 136.99, 140.24, 140.75, 142.62, 148.94, 155.36, 164.52, 189.15. ESIMS $[M+H]^+$: 373.08. Anal. calc. for $C_{22}H_{20}N_4O_2 \cdot 0.2\ H_2O$: C 70.26, H 5.48, N 14.90; found: C 69.98, H 5.50, N 14.76.

**5.4.4. *N*-(2-Morpholinoethyl)-11-oxo-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide (9d).** green solid (0.48 g, 60% yield); Mp: 217-219 °C (from EtOH, decomp.). $^1$H NMR (400 MHz, $CDCl_3$): δ 2.62 (t, 2H, *J* = 4.8 Hz, morpholinyl-H), 2.78 (t, 2H, *J* = 6.0 Hz, $NHCH_2CH_2N$), 3.77 (t, 2H, *J* = 4.8 Hz, morpholinyl-H), 3.80 (q, 2H, *J* = 6.0 Hz, $NHCH_2CH_2N$), 7.68 (td, 1H, *J* = 1.2, 7.6 Hz, ArH), 7.78 (td, 1H, *J* = 7.6, 1.2 Hz, ArH), 7.86 (t, 1H, *J* = 7.6 Hz, ArH), 7.97 (d, 1H, *J* = 7.6 Hz, ArH), 8.17 (d, 1H, *J* = 7.2 Hz, ArH), 8.37 (dd, 1H, *J* = 8.0, 1.6 Hz, ArH), 8.91 (d, 1H, *J* = 1.6, 7.6 Hz, ArH), 10.42 (br, 1H, CONH). $^{13}$C-NMR (100 MHz, $CDCl_3$): δ 36.86, 53.80 (2C), 57.94, 66.82 (2C), 122.54, 125.20, 129.94, 130.12, 133.48, 135.36, 136.62, 136.67, 137.03, 140.15, 140.53, 142.70, 148.94, 155.30, 164.61, 189.00. ESIMS $[M+H]^+$: 389.10. Anal. calc. for $C_{22}H_{20}N_4O_3$: C 68.02, H 5.20, N 14.43; found: C 67.74, H 5.25, N 14.38.

**5.5.1. 11-{[3-(Dimethylamino)propoxy]imino}-*N*-[3-(dimethylamino)propyl]-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide hydrochloride (10a).**

A mixture of **9a** (0.36, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) in EtOH (5.0 mL) was refluxed for 6 h (TLC monitoring). The mixture was cooled and concentrated. The residue was dissolved in water (20 mL), neutralized by $K_2CO_3$ (0.41 g, 3.0 mmol), and extracted with $CH_2Cl_2$ three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH/$NH_4OH$ 100/20/1). The oil compound was stirred with 3N HCl (3.0 mL) in EtOH (2.0 mL) for 0.5 h, then evaporated in vecuo. The residue crystallized from EtOH to give **10a** (0.45 g, 84% yield) as a white solid. Mp: 228-230 °C (from EtOH, decomp.). $^1$H NMR (400 MHz, $D_2O$): δ 1.84-1.92 (m, 2H,

NHCH$_2$CH$_2$CH$_2$N), 2.14-2.21 (m, 2H, OCH$_2$CH$_2$CH$_2$N), 2.91 (s, 6H, N(CH$_3$)$_2$), 2.92 (s, 6H, N(CH$_3$)$_2$), 3.16-3.28 (m, 6H, NHCH$_2$CH$_2$CH$_2$N, OCH$_2$CH$_2$CH$_2$N), 4.21 (t, 2H, *J* = 6.0 Hz, OCH$_2$CH$_2$CH$_2$N), 7.09-7.11 (m, 1H, ArH), 7.33-7.46 (m, 2H, ArH), 7.49-7.61 (m, 3H, ArH), 7.97 (dd, 1H, *J* = 7.2, 1.2 Hz, ArH). $^{13}$C NMR (100 MHz, D$_2$O): δ 24.08, 24.29, 37.00, 42.99 (2C), 43.12 (2C), 54.98, 55.49, 73.39, 121.84, 126.92, 128.85, 129.72, 131.78, 132.98, 133.57, 133.66, 133.74, 133.84, 137.99, 139.84, 145.53, 148.35, 150.54, 165.80. ESIMS [M+H]$^+$: 461.21. Anal. calc. for C$_{26}$H$_{32}$N$_6$O$_2$ · 2.0 HCl · 3.0 H$_2$O: C 53.13, H 6.87, N 14.30; found: C 52.90, H 6.89, N 14.22.

**5.5.2. *N*-[2-(Dimethylamino)ethyl]-11-{[3-(dimethylamino)propoxy]imino}-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide hydrochloride (10b).**

Compound **10b** was obtained from **9b** (0.35 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.61 g) as described for the preparation of **10a** in 89% yield as a white solid. Mp: 244-245 °C (from EtOH, decomp.). $^1$H NMR (400 MHz, D$_2$O): δ 2.14-2.21 (m, 2H, OCH$_2$CH$_2$CH$_2$N), 2.89 (s, 6H, N(CH$_3$)$_2$), 2.94 (s, 6H, N(CH$_3$)$_2$), 3.23-3.27 (m, 2H, OCH$_2$CH$_2$CH$_2$N), 3.31 (t, 2H, *J* = 6.4 Hz, NHCH$_2$CH$_2$N), 3.60 (t, 2H, *J* = 6.4 Hz, NHCH$_2$CH$_2$N), 4.27 (t, 2H, *J* = 6.4 Hz, OCH$_2$CH$_2$CH$_2$N), 7.37-7.39 (m, 1H, ArH), 7.41-7.49 (m, 2H, ArH), 7.53 (t, 1H, *J* = 7.6 Hz, ArH), 7.63-7.66 (m, 2H, ArH), 8.04 (dd, 1H, *J* = 7.6, 1.2 Hz, ArH). $^{13}$C NMR (100 MHz, D$_2$O): δ 24.14, 35.15, 42.94 (2C), 43.44 (2C), 54.95, 56.73, 73.36, 122.35, 126.93, 128.84, 129.68, 131.91, 133.02, 133.66, 133.81, 133.91, 134.10, 138.25, 139.88, 145.86, 148.68, 151.06, 166.88. ESIMS [M+H]$^+$: 447.09. Anal. calc. for C$_{25}$H$_{30}$N$_6$O$_2$ · 2.0 HCl · 2.8 H$_2$O: C 52.67, H 6.66, N 14.75; found: C 52.52, H 6.51, N 14.41.

**5.5.3. 11-{[3-(Dimethylamino)propoxy]imino}-*N*-[2-(pyrrolidin-1-yl)ethyl]-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide hydrochloride (10c).**

Compound **10c** was obtained from **9c** (0.37 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) as described for the preparation of **10a** in 82% yield as a white solid. Mp: 226-227 °C (from EtOH, decomp.). $^1$H NMR (400

MHz, $D_2O$): δ 2.01 (br, 4H, pyrrolidinyl-H), 2.13-2.20 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.86 (s, 6H, $N(\underline{CH_3})_2$), 3.21-3.25 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 3.33-3.39 (m, 6H, $NHCH_2\underline{CH_2}N$, pyrrolidinyl-H), 3.56 (t, 2H, *J* = 6.0 Hz, $NH\underline{CH_2}CH_2N$), 4.26 (t, 2H, *J* = 6.0 Hz, $O\underline{CH_2}CH_2CH_2N$), 7.35 (d, *J* = 6.8 Hz, ArH), 7.41-7.47 (m, 2H, ArH), 7.51 (t, 1H, *J* = 8.0 Hz, ArH), 7.61 (d, 1H, *J* = 8.0 Hz, ArH), 7.65 (d, 1H, *J* = 6.4 Hz, ArH), 8.03 (d, 1H, *J* = 7.2 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 22.84 (2C), 24.16, 36.24, 42.93 (2C), 53.91, 54.81 (2C), 54.95, 73.37, 122.30, 127.02, 128.86, 129.68, 131.97, 133.01, 133.66, 133.77, 133.88, 134.15, 138.28, 139.91, 145.93, 148.73, 151.10, 166.79. ESIMS $[M+H]^+$: 473.12. Anal. calc. for $C_{27}H_{32}N_6O_2 \cdot 2.0\ HCl \cdot 2.8\ H_2O$: C 54.40, H 6.71, N 14.10; found: C 54.11, H 6.75, N 14.03.

**5.5.4. 11-{[3-(Dimethylamino)propoxy]imino}-*N*-(2-morpholinoethyl)-11*H*-indeno[1,2-*b*]quinoxaline-6-carboxamide hydrochloride (10d).**

Compound **10d** was obtained from **9d** (0.39 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) as described for the preparation of **10a** in 73% yield as a green solid. Mp: 227-229 $^{o}$C (from EtOH, decomp.). $^1$H NMR (400 MHz, $D_2O$): δ 2.14-2.20 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 3.21-3.25 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 3.33-3.42 (m, 6H, $NHCH_2\underline{CH_2}N$, morpholinyl-H), 3.64 (t, 2H, *J* = 6.8 Hz, $NH\underline{CH_2}CH_2N$), 3.95 (br, 4H, morpholinyl-H), 4.25 (t, 2H, *J* = 6.4 Hz, $O\underline{CH_2}CH_2CH_2N$), 7.40-7.49 (m, 3H, ArH), 7.53 (t, 1H, *J* = 7.6 Hz, ArH), 7.62-7.65 (m, 2H, ArH), 8.05 (dd, 1H, *J* = 7.6, 1.2 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 24.15, 34..46, 42.95 (2C), 52.42 (2C), 54.97, 56.20, 63.99 (2C), 73.37, 122.40, 127.06, 128.85, 129.66, 131.96, 133.01, 133.67, 133.87, 133.92, 134.19, 138.31, 139.95, 145.88, 148.74, 151.12, 167.03. ESIMS $[M+H]^+$: 489.17. Anal. calc. for $C_{27}H_{32}N_6O_3 \cdot 2.0\ HCl \cdot 2.6\ H_2O$: C 53.29, H 6.51, N 13.81; found: C 53.01, H 6.60, N 13.78.

**5.6. 6-(Bromomethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one (11).**

A mixture of **4b** (2.46 g, 10.0 mmol), *N*-bromosuccinimide (1.96 g, 11.0 mmol) and dibenzoylperoxide (0.24 g, 1.0 mmol) in $CCl_4$ (50 mL) was refluxed for 2 h (TLC

monitoring). After cooling, EtOH (30 mL) was added and stirred for 10 min. The resulting precipitate was filtered, washed with EtOH (20 mL), and crystallized from EtOH/$CH_2Cl_2$ (4/1) to give **11** (2.99 g, 92% yield) as a yellow solid. Mp: 258-260 $^oC$ (from EtOH, decomp.). $^1H$ NMR (400 MHz, $CDCl_3$): δ 5.24 (s, 2H, Ar$\underline{CH_2}$Br), 7.60-7.64 (m, 1H, ArH), 7.69-7.73 (m, 1H, ArH), 7.77-7.81 (m, 1H, ArH), 7.92-7.94 (m, 2H, ArH), 8.16 (d, 1H, *J* = 7.6 Hz, ArH), 8.19 (dd, 1H, *J* = 8.4, 1.2 Hz, ArH). $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 27.71, 122.83, 124.78, 129.99, 132.14, 132.67, 133.44, 136.73, 136.86, 137.15, 140.82, 141.53, 142.69, 149.60, 156.08, 189.81. Anal. calc. for $C_{16}H_9N_2OBr$: C 59.10, H 2.80, N 8.62; found: C 59.36, H 2.93, N 8.73.

**5.7. 11-Oxo-11*H*-indeno[1,2-*b*]quinoxaline-6-carbaldehyde (12).**

A mixture of **11** (1.63 g, 5.0 mmol) and calcium carbonate (15.0 g, 150 mmol) in 1,4-dioxane/water (4/1, 50 mL) was refluxed for 2 days (TLC monitoring). After cooling, the mixture was filtered and washed with 1,4-dioxane (20 mL). The filtrate was evaporated in vacuo to give yellow solid, then Manganese(IV) oxide (8.69 g, 100.0 mmol) and $CH_2Cl_2$ (100 mL) was added and stirred at room temperature for 12 h. The mixture was filtered by celite and washed with $CH_2Cl_2$ (50 mL). The filtrate was evaporated in vacuo and purified by column chromatography ($CH_2Cl_2$) and crystallized from EtOH to give **12** (1.20 g, 92% yield) as a yellow solid. Mp: 270-272 $^oC$ (from EtOH, decomp.). $^1H$ NMR (400 MHz, $CDCl_3$): δ 7.65 (t, 1H, *J* = 7.6 Hz, ArH), 7.87 (t, 1H, *J* = 7.6 Hz, ArH), 7.86 (t, 1H, *J* = 7.6 Hz, ArH), 7.94 (d, 1H, *J* = 7.6 Hz, ArH), 8.13 (d, 1H, *J* = 7.6 Hz, ArH), 8.39 (dd, 1H, *J* = 7.6, 1.2 Hz, ArH), 8.39 (dd, 1H, *J* = 8.0, 1.2 Hz, ArH), 11.41 (s, 1H, ArH). $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 122.91, 124.92, 129.77, 131.42, 132.23, 133.24, 136.93, 137.08, 137.48, 140.97, 142.11, 142.84, 149.89, 157.09, 189.22, 190.61. ESIMS $[M+H]^+$: 260.92. Anal. calc. for $C_{16}H_8N_2O_2$: C 73.83, H 3.10, N 10.77; found: C 73.64, H 3.25, N 10.59.

**5.8.1. 11-(Hydroxyimino)-11*H*-indeno[1,2-*b*]quinoxaline-6-carbaldehyde oxime (13).**

Compound **13** was obtained from **12** (1.0 mmol) and hydroxylamine hydrochloride (8.0 mmol) as described for the preparation of **3** in 87% yield as a white solid. Mp: 290-291 $^oC$

(from EtOH, decomp.). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 7.71-7.76 (m, 2H, ArH), 7.82 (t, 1H, *J* = 8.0 Hz, ArH), 8.15 (dd, 1H, *J* = 8.0, 1.2 Hz, ArH), 8.21-8.25 (m, 2H, ArH), 8.53-8.55 (m, 1H, ArH), 9.28 (s, 1H, CHN), 11.68 (s, 1H, NOH), 13.43 (s, 1H, NOH). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 122.22, 125.51, 128.54, 129.40, 130.65, 130.69, 131.84, 132.50, 133.13, 135.69, 139.10, 141.30, 144.14, 146.91, 150.98, 152.09. ESIMS $[M+H]^+$: 290.93. Anal. calc. for $C_{16}H_{10}N_4O_2$: C 66.19, H 3.48, N 19.30; found: C 66.23, H 3.52, N 19.20.

**5.8.2. 11-{[2-(Pyrrolidin-1-yl)ethoxy]imino}-11*H*-indeno[1,2-*b*]quinoxaline-6-carbaldehyde *O*-[2-(pyrrolidin-1-yl)ethyl] oxime hydrochloride (14).**

A mixture of **12** (0.26 g, 1.0 mmol) and 2-(pyrrolidino)propoxyamine hydrochloride (1.20 g) in EtOH (5.0 mL) was refluxed for 6 h. The mixture was cooled and concentrated, then dissolved in water (20 mL), neutralized by $K_2CO_3$ (0.83 g, 6.0 mmol), and extracted with $CH_2Cl_2$ three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH/$NH_4OH$ 100/20/1). The oil compound was stirred with 3N HCl (3.0 mL) in EtOH (2.0 mL) for 0.5 h, then evaporated in vecuo. The residue was dissolved in EtOH (2.0 mL), then poured into acetone (50 mL). The resulting precipitate was filtered and dried to give **14** (0.38 g, 68% yield) as a green solid. Mp: 217-218 $^{o}$C (decomp.). $^{1}$H NMR (400 MHz, $D_2O$): δ 1.96-2.20 (m, 8H, pyrrolindinyl-H), 3.13-3.23 (m, 4H, pyrrolindinyl-H), 3.62-3.76 (m, 8H, $(OCH_2CH_2N)_2$, pyrrolindinyl-H), 4.50-4.62 (m, 4H, $(OCH_2CH_2N)_2$), 7.28-7.40 (m, 3H, ArH), 7.45-7.55 (m, 2H, ArH), 7.73-7.82 (m, 2H, ArH), 8.47 (s, 1H, CHN). $^{13}$C NMR (100 MHz, $D_2O$): δ 19.98 (4C), 50.97, 51.05, 52.07 (2C), 52.31 (2C), 66.34, 68.82, 119.24, 124.83, 125.43, 126.18, 127.15, 128.25, 128.87, 130.25 (2C), 132.52, 136.24, 136.75, 144.38, 144.63, 146.11, 149.17. ESIMS $[M+H]^+$: 485.18.Anal. calc. for $C_{28}H_{32}N_6O_2 \cdot 2.0\ HCl \cdot 2.8\ H_2O$: C 55.30, H 6.58, N 13.82; found: C 54.91, H 6.58, N 13.61.

**5.8.3. 11-{[3-(Dimethylamino)propoxy]imino}-11*H*-indeno[1,2-*b*]quinoxaline-6-carbaldehyde *O*-[3-(dimethylamino)propyl] oxime hydrochloride (15).**

Compound **15** was obtained from **12** (0.26 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (1.20 g) as described for the preparation of **14** in 60% yield as a white solid. Mp: 267-269 °C (decomp.). $^1$H NMR (400 MHz, $D_2O$): δ 2.11-2.16 (m, 4H, ($OCH_2\underline{CH_2}CH_2N)_2$), 2.86 (s, 6H, $N(\underline{CH_3})_2$), 2.89 (s, 6H, $N(\underline{CH_3})_2$), 3.18-3.29 (m, 4H, ($OCH_2CH_2\underline{CH_2}N)_2$), 4.17-4.23 (m, 4H, ($O\underline{CH_2}CH_2CH_2N)_2$), 7.02-7.08 (m, 1H, ArH), 7.14-7.20 (m, 2H, ArH), 7.29-7.38 (m, 2H, ArH), 7.43-7.50 (m, 2H, ArH), 8.06 (s, 1H, C<u>H</u>N). $^{13}$C NMR (100 MHz, $D_2O$): δ 24.20 (2C), 42.95 (2C), 43.02 (2C), 55.00, 55.23, 70.85, 73.02, 121.75, 126.92, 128.03, 128.49, 129.62, 130.91, 131.50, 132.50, 132.75, 134.72, 138.60, 139.41, 146.05, 146.19, 148.65, 151.38. ESIMS $[M+H]^+$: 461.15. Anal. calc. for $C_{26}H_{32}N_6O_2 \cdot 3.0\ HCl \cdot 0.7\ H_2O$: C 53.58, H 6.31, N 14.42; found: C 53.31, H 6.35, N 14.37.

**5.9.1. 6-[(Dimethylamino)methyl]-11*H*-indeno[1,2-*b*]quinoxalin-11-one (16a).**

Compound **11** (0.98 g, 3.0 mmol) was stirred in THF (20 mL) at room temperature, then dimethylamine (40% in water, 1.0 mL, 9.0 mmol) was added, and the reaction mixture was stirred for 10 min (TLC monitoring). The solvent evaporated in vecuo, purified by column chromatography ($CH_2Cl_2$/MeOH 10/1) and crystallized from EtOH to give **16a** (0.62 g, 71% yield) as a yellow solid. Mp: 171-173 °C (from EtOH, decomp.). $^1$H NMR (400 MHz, $CDCl_3$): δ 2.50 (s, 6H, $N(\underline{CH_3})_2$), 4.33 (s, 2H, $Ar\underline{CH_2}N$), 7.58 (td, 1H, *J* = 0.8, 7.6 Hz, ArH), 7.73-7.79 (m, 2H, ArH), 7.90 (d, 1H, *J* = 7.2 Hz, ArH), 7.98 (d, 1H, *J* = 7.2 Hz, ArH), 8.11 (d, 1H, *J* = 7.6 Hz, ArH), 8.17 (dd, 1H, *J* = 7.6, 1.2 Hz, ArH). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 45.11 (2C), 57.09, 122.57, 124.68, 129.89, 131.25, 132.47, 133.71, 136.07, 136.04, 136.67, 141.63, 141.85, 142.65, 149.07, 155.91, 189.95. ESIMS $[M+H]^+$: 290.05. Anal. calc. for $C_{18}H_{15}N_3O \cdot 0.6\ H_2O$: C 72.02, H 5.45, N 14.00; found: C 71.98, H 5.18, N 13.88.

**5.9.2. 6-(Pyrrolidin-1-ylmethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one hydrobromide (16b).**

Compound **11** (0.98 g, 3.0 mmol) was stirred in $CH_2Cl_2$ (20 mL) at room temperature, then pyrrolidine (0.43 g, 6.0 mmol) was added, and the reaction mixture was stirred for 10 min

(TLC monitoring). The mixture was extracted with water three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH 10/1) to give **16b** (0.69 g, 58% yield) as a green solid. Mp: 268-270 $^oC$ (decomp.). $^1H$ NMR (400 MHz, $CDCl_3$): δ 2.15 (br, 2H, pyrrolidinyl-H), 2.29 (br, 2H, pyrrolidinyl-H), 3.12 (br, 2H, pyrrolidinyl-H), 3.78 (br, 2H, pyrrolidinyl-H), 5.08 (s, 2H, Ar$\underline{CH_2}$N), 7.65 (t, 1H, *J* = 7.2 Hz, ArH), 7.80 (td, 1H, *J* = 7.6, 1.2 Hz, ArH), 7.85 (t, 1H, *J* = 7.6 Hz, ArH), 7.94 (d, 1H, *J* = 7.2 Hz, ArH), 8.15 (d, 1H, *J* = 7.6 Hz, ArH), 8.30 (dd, 1H, *J* = 8.0, 0.8 Hz, ArH), 8.62 (d, 1H, *J* = 7.2 Hz, ArH). $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 23.13 (2C), 51.04, 53.32 (2C), 122.79, 125.00, 128.54, 130.49, 133.22, 133.70, 136.76, 136.87, 136.99, 140.82, 141.34, 142.59, 149.82, 156.51, 189.08. ESIMS $[M+H]^+$: 316.03. Anal. calc. for $C_{20}H_{17}N_3O \cdot 0.9$ HBr: C 60.60, H 4.56, N 10.60; found: C 60.56, H 4.61, N 10.40.

**5.9.3. 6-(Piperidin-1-ylmethyl)-11H-indeno[1,2-*b*]quinoxalin-11-one (16c).**

Compound **16c** was obtained from **11** (0.98 g, 3.0 mmol) and piperidine (0.51 g, 6.0 mmol) as described for the preparation of **16b**. Compound was crystallized from EtOH in 62% yield as a yellow solid. Mp: 175-176 $^oC$ (from EtOH, decomp.). $^1H$ NMR (400 MHz, $CDCl_3$): δ 1.44 (quin, 2H, *J* = 5.6 Hz, piperidinyl-H), 1.61-1.67 (quin, 4H, *J* = 5.6 Hz, piperidinyl-H), 2.56 (br, 4H, piperidinyl-H), 4.25 (s, 2H, Ar$\underline{CH_2}$N), 7.56 (td, 1H, *J* = 7.6, 0.8 Hz, ArH), 7.70-7.77 (m, 2H, ArH), 7.89-7.92 (m, 1H, ArH), 7.95 (dd, 1H, *J* = 7.2, 0.8 Hz, ArH), 8.10-8.13 (m, 2H, ArH). $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 24.29, 26.09 (2C), 54.80 (2C), 57.00, 122.44, 124.63, 129.89, 130.19, 132.24, 132.65, 136.00 (2C), 138.09, 141.89 (2C), 142.56, 148.75, 155.50, 190.27. ESIMS $[M+H]^+$: 330.09. Anal. calc. for $C_{21}H_{19}N_3O \cdot 0.3$ $H_2O$: C 75.32, H 5.91, N 12.55; found: C 75.13, H 5.91, N 12.41.

**5.9.4. 6-(Morpholinomethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one (16d).**

Compound **16d** was obtained from **11** (0.98 g, 3.0 mmol) and morpholine (0.52 g, 6.0 mmol) as described for the preparation of **16b** in 72% yield as a yellow solid. Mp: 237-238 $^oC$ (from EtOH, decomp.). $^1H$ NMR (400 MHz, $CDCl_3$): δ 2.64 (t, 4H, *J* = 4.8 Hz, N($\underline{CH_2}CH_2)_2$O), 3.76 (t, 4H, *J* = 4.8 Hz, N($CH_2\underline{CH_2})_2$O), 4.29 (s, 2H, Ar$\underline{CH_2}$N), 7.58 (td, 1H,

*J* = 7.6, 0.8 Hz, ArH), 7.71-7.79 (m, 2H, ArH), 7.91 (d, 1H, *J* = 7.6 Hz, ArH), 7.96 (dd, 1H, *J* = 7.2, 0.8 Hz, ArH), 8.10 (d, 1H, *J* = 7.2 Hz, ArH), 8.13 (dd, 1H, *J* = 7.6, 1.2 Hz, ArH). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 53.80 (2C), 56.62, 67.06 (2C), 122.47, 124.69, 129.86, 130.54, 132.37, 132.68, 136.62, 136.68, 137.13, 141.75, 141.85, 142.59, 148.91, 155.62, 190.14. ESIMS $[M+H]^+$: 332.04. Anal. calc. for $C_{20}H_{17}N_3O_2$: C 72.48, H 5.18, N 12.68; found: C 72.23, H 5.28, N 12.69.

**5.9.5. 6-[(1,4'-Bipiperidin)-1'-ylmethyl]-11*H*-indeno[1,2-*b*]quinoxalin-11-one (16e).**

Compound **16e** was obtained from **11** (0.98 g, 3.0 mmol) and *N*-(4-piperidino)piperidine (1.01 g, 6.0 mmol) as described for the preparation of **16b** in 60% yield as a green solid. Mp: 198-200 $^{o}$C (from EtOH, decomp.). $^1$H NMR (400 MHz, $CDCl_3$): δ 1.45-1.47 (m, 2H, piperidinyl-H), 1.61-1.87 (m, 8H, piperidinyl-H), 2.17-2.23 (m, 2H, piperidinyl-H), 2.36-2.42 (m, 1H, piperidinyl-H), 2.52 (br, 4H, piperidinyl-H), 3.09-3.12 (m, 2H, piperidinyl-H), 4.26 (s, 2H, Ar$\underline{CH_2}$N), 7.57 (td, 1H, *J* = 7.6, 1.2 Hz, ArH), 7.70-7.78 (m, 2H, ArH), 7.90-7.95 (m, 2H, ArH), 8.09-8.14 (m, 2H, ArH). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 24.54, 25.96 (2C), 27.74 (2C), 50.10 (2C), 53.61 (2C), 56.15, 62.82, 122.44, 124.64, 129.90, 130.29, 132.28, 132.55, 136.62 (2C), 137.98, 141.81 (2C), 142.57, 148.81, 155.53, 190.20. ESIMS $[M+H]^+$: 413.11. Anal. calc. for $C_{26}H_{28}N_4O \cdot 0.2\ H_2O$: C 75.03, H 6.89, N 13.47; found: C 74.95, H 6.85, N 13.40.

**5.10.1. 6-[(Dimethylamino)methyl]-11*H*-indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethylamino)propyl] oxime hydrochloride (17a).**

A mixture of **16a** (0.29 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.61 g) in EtOH (5.0 mL) was refluxed for 6 h (TLC monitoring). The mixture was cooled and concentrated, then dissolved in water (20 mL), neutralized by $K_2CO_3$ (0.41 g, 3.0 mmol), and extracted with $CH_2Cl_2$ three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH/$NH_4OH$ 100/20/1). The oil compound was stirred with 3N HCl (3.0 mL) in EtOH (2.0 mL) for 0.5 h, then evaporated in vecuo. The residue was dried to give **17a** (0.31 g, 57% yield) as a green solid. Mp: 234-236 $^{o}$C

(decomp.). $^1$H NMR (400 MHz, $D_2O$): δ 2.21-2.25 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.82 (s, 6H, $N(\underline{CH_3})_2$), 2.85 (s, 6H, $N(\underline{CH_3})_2$), 3.24-3.28 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 4.41 (t, 2H, *J* = 5.6 Hz, $O\underline{CH_2}CH_2CH_2N$), 4.51 (s, 2H, $Ar\underline{CH_2}N$), 7.45-7.53 (m, 2H, ArH), 7.63 (t, 1H, *J* = 7.6 Hz, ArH), 7.73-7.80 (m, 3H, ArH), 7.88 (d, 1H, *J* = 7.2 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 24.32, 42.90 (2C), 43.01 (2C), 54.99, 57.27, 73.23, 122.45, 127.58, 128.93, 130.13, 131.26, 132.10, 132.95, 133.25, 134.24, 135.44, 140.25, 140.31, 146.98, 149.92, 152.68. ESIMS $[M+H]^+$: 390.10. Anal. calc. for $C_{23}H_{27}N_5O \cdot 4.0$ HCl: C 51.58, H 5.85, N 13.08; found: C 51.55, H 5.84, N 13.20.

**5.10.2. 6-(Pyrrolidin-1-ylmethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethylamino)propyl] oxime hydrochloride (17b).**

A mixture of **16b** (0.32 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) in EtOH (5.0 mL) was refluxed for 6 h. The mixture was cooled and concentrated, then dissolved in water (20 mL), neutralized by $K_2CO_3$ (0.41 g, 3.0 mmol), and extracted with $CH_2Cl_2$ three times. The organic layer evaporated in vecuo and purified by column chromatography ($CH_2Cl_2$/MeOH/$NH_4OH$ 100/20/1). The oil compound was stirred with 3N HCl (3.0 mL) in EtOH (2.0 mL) for 0.5 h, then evaporated in vecuo. The residue was dissolved in EtOH (2 mL), then poured into acetone (50 mL). The resulting precipitate was filtered and dried to give **17b** (0.32 g, 66% yield) as a green solid. Mp: 113-114 $^o$C. $^1$H NMR (400 MHz, $D_2O$): δ 1.89-1.95 (m, 2H, pyrrolidinyl-H), 2.08-2.14 (m, 2H, pyrrolidinyl-H), 2.18-2.25 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.87 (s, 6H, $N(\underline{CH_3})_2$), 3.18-3.28 (m, 4H, $OCH_2CH_2\underline{CH_2}N$, pyrrolidinyl-H), 3.39-3.45 (m, 2H, pyrrolidinyl-H), 4.36 (t, 2H, *J* = 6.0 Hz, $O\underline{CH_2}CH_2CH_2N$), 4.54 (s, 2H, $Ar\underline{CH_2}N$), 7.40-7.48 (m, 2H, ArH), 7.57-7.61 (m, 1H, ArH), 7.66-7.73 (m, 3H, ArH), 7.80 (dd, 1H, *J* = 6.4, 1.2 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 22.70 (2C), 24.30, 42.94 (2C), 53.56, 54.47 (2C), 55.02, 73.20, 122.32, 128.53, 128.89, 130.08, 131.00, 132.02, 132.86, 133.13, 133.77, 135.46, 140.13, 140.21, 146.88, 149.72, 152.60. ESIMS $[M+H]^+$: 416.14. Anal. calc. for $C_{25}H_{29}N_5O \cdot 2.0$ HCl $\cdot$ 3.3 $H_2O$: C 54.78, H 6.93, N 12.78; found: C 54.47, H 6.98, N 12.69.

**5.10.3. 6-(Piperidin-1-ylmethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethyl-amino)propyl] oxime (17c).**

Compound **17c** was obtained from **16c** (0.33 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g) as described for the preparation of **17b** in 74% yield as a green solid. Mp: 218-220 $^{o}$C (decomp.). $^{1}$H NMR (400 MHz, $D_2O$): δ 1.39-1.69 (m, 4H, piperidinyl-H), 1.82-1.85 (m, 2H, piperidinyl-H), 2.17-2.24 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.86 (s, 6H, $N(\underline{CH_3})_2$), 2.95-3.02 (m, 2H, piperidinyl-H), 3.22-3.27 (m, 2H, $OCH_2CH_2\underline{CH_2}N$), 3.35-3.39 (m, 2H, piperidinyl-H), 4.35 (t, 2H, *J* = 6.4 Hz, $O\underline{CH_2}CH_2CH_2N$), 4.46 (s, 2H, $Ar\underline{CH_2}N$), 7.41-7.50 (m, 2H, ArH), 7.57-7.61 (m, 1H, ArH), 7.65-7.67 (m, 2H, ArH), 7.70 (dd, 1H, *J* = 6.8, 1.2 Hz, ArH), 7.81-7.83 (m, 1H, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 21.14, 22.62 (2C), 24.31, 42.95 (2C), 53.48 (2C), 55.04 (2C), 73.21, 122.25, 127.34, 128.92, 130.00, 131.17, 132.09, 132.92, 133.17, 134.47, 135.53, 140.27, 140.58, 146.94, 149.69, 152.62. ESIMS $[M+H]^+$: 430.13. Anal. calc. for $C_{26}H_{31}N_5O \cdot 2.0\ HCl \cdot 2.9\ H_2O$: C 56.28, H 7.06, N 12.62; found: C 55.91, H 7.04, N 12.54.

**5.10.4. 6-(Morpholinomethyl)-11*H*-indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethyl-amino)propyl] oxime hydrochloride (17d).**

Compound **17d** was obtained from **16d** (0.33 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.61 g) as described for the preparation of **17b** in 61% yield as a green solid. Mp: 224-226 $^{o}$C (decomp.). $^{1}$H NMR (400 MHz, $CDCl_3$): δ 2.19-2.26 (m, 2H, $OCH_2\underline{CH_2}CH_2N$), 2.88 (s, 6H, $N(\underline{CH_3})_2$), 3.25-3.29 (m, 4H, $OCH_2CH_2\underline{CH_2}N$, morpholinyl-H), 3.38-3.41 (m, 2H, morpholinyl-H), 3.66-3.72 (m, 2H, morpholinyl-H), 3.99-4.03 (m, 2H, morpholinyl-H), 4.36 (t, 2H, *J* = 6.0 Hz, $O\underline{CH_2}CH_2CH_2N$), 4.60 (s, 2H, $Ar\underline{CH_2}N$), 7.43-7.52 (m, 2H, ArH), 7.60-7.65 (m, 2H, ArH), 7.70 (d, 1H, *J* = 6.8 Hz, ArH), 7.76-7.80 (m, 2H, ArH). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 24.28, 42.95 (2C), 51.92 (2C), 55.03, 55.12, 63.71 (2C), 73.19, 122.32, 126.47, 128.92, 130.04, 131.45, 132.05, 132.91, 133.20, 134.82, 135.47, 140.24, 140.57, 146.87, 149.68, 152.69. ESIMS $[M+H]^+$: 432.15. Anal. calc. for $C_{25}H_{29}N_5O_2 \cdot 2.0\ HCl \cdot 4.6\ H_2O$: C 51.11, H

6.91, N 11.92; found: C 50.82, H 6.98, N 11.84.

**5.10.5. 6-[(1,4'-Bipiperidin)-1'-ylmethyl]-11*H*-indeno[1,2-*b*]quinoxalin-11-one *O*-[3-(dimethylamino)propyl] oxime hydrochloride (17e).**

Compound **17e** was obtained from **16e** (0.41 g, 1.0 mmol) and 3-(dimethylamino)propoxyamine hydrochloride (0.60 g mmol) as described for the preparation of **17b** in 72% yield as a white solid. Mp: 225-227 $^{o}$C (decomp.). $^{1}$H NMR (400 MHz, $D_2O$): δ 1.37-1.89 (m, 7H, piperidinyl-H), 2.22-2.29 (m, 4H, $OCH_2\underline{CH_2}CH_2N$, piperidinyl-H), 2.85 (s, 6H, $N(\underline{CH_3})_2$), 2.90-3.00 (m, 2H, piperidinyl-H), 3.14-3.30 (m, 5H, $OCH_2CH_2\underline{CH_2}N$, piperidinyl-H), 3.40-3.63 (m, 5H, piperidinyl-H), 4.46 (t, 2H, *J* = 6.0 Hz, $O\underline{CH_2}CH_2CH_2N$), 4.61 (s, 2H, $Ar\underline{CH_2}N$), 7.50-7.60 (m, 2H, ArH), 7.69-7.85 (m, 3H, ArH), 7.88 (dd, 1H, *J* = 8.4, 1.6 Hz, ArH), 7.97 (d, 1H, *J* = 7.2 Hz, ArH). $^{13}$C NMR (100 MHz, $D_2O$): δ 21.27, 23.09 (2C), 24.40, 42.95 (2C), 50.59 (2C), 51.09, 55.06 (2C), 55.19, 60.01, 73.30, 122.52, 126.98, 129.04, 130.07, 131.60, 132.30, 133.08, 133.27, 134.70, 135.77, 140.46, 140.72, 147.24, 150.06, 153.02. ESIMS $[M+H]^+$: 513.27. Anal. calc. for $C_{31}H_{40}N_6O \cdot 3.0\ HCl \cdot 4.0\ H_2O$: C 53.62, H 7.42, N 12.11; found: C 53.29, H 7.45, N 11.97.

**5.11. Pharmacological methods**

**5.11.1. Antiproliferative Assay.**

Cancer cells (MDA-MB231, H1299, PC-3, Huh-7) and normal mammary lung cell (MRC-5) were purchased from Bioresources Collection and Research Center, Taiwan. Cell lines were maintained in the same standard medium and grown as a monolayer in DMEM (Gibco,USA) and supplemented with 10% fetal bovine serum (FBS) and antibiotics i.e. 100 IU/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotercin. Culture was maintained at 37°C with 5% $CO_2$ in a humidified atmosphere.

Cells (5 x $10^3$ cells/well) were treated as indicated for 72 h in medium containing 10% FBS. Cell viability was quantitated with the use of sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) colorimetric assay (Biological Industries, Beit-Haemek, Israel). XTT labeling reagent (1 mg/mL) was

mixed with electron-coupling reagent, following the manufacturer's instructions, and 50 µL of the mixture was added directly to the cells. The plates were further incubated at 37°C for 4 h. Color was measured spectrophotometrically in a microtiter plate reader at 492 nm and used as a relative measure of viable cell number. The number of viable cells following treatment was compared to solvent and untreated control cells and used to determine the percent of control growth as ($Ab_{treated}$/ $Ab_{control}$) x100, where Ab represents the mean absorbance (n = 3). The concentration that killed 50% of cells ($GI_{50}$) was determined from the linear portion of the curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells, by 50% [22].

**5.11.2. DNA mobility assay (DNA unwinding).**

Negative supercoiled pBR322 (400 ng) was incubated in TE buffer, pH = 8.0, with 10 µM of different indeno[1,2-*b*] quinoxaline derivatives for 12 h at room temperature. Following the addition of 2 µL of loading buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol), the samples were loaded onto a 1% agarose gel. The gel was run at 6 V $cm^{-1}$ for 2.5 h in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA-$Na_2$, pH = 8.5) and stained with ethidium bromide and photographed under UV illumination using a Polaroid instant film [16].

**5.11.3. Topoisomerases (topo I and topo II) Inhibitory Assay**

Type I or type II DNA topoisomerase was assayed by measuring the decreased mobility of the relaxed isomers of supercoiled pBR322 DNA in an agarose gel after it had been treated with human topo I or II. The standard topoisomerase assay mixture (20 µl) contained: 40 mM of Tris-HCl (pH 7.5), 100 mM of KCl, 10 mM of DTT, 0.5 mM of EDTA, 10 mM of $MgCl_2$, 30 µg/ml of BSA, 0.2 µg of pBR322 DNA, and two units of enzyme (one unit is defined as the amount of enzyme required to convert 0.2 µg of supercoiled DNA substrate to the relaxed form under standard assay conditions). Reactions were done at 37°C for 30 minutes, and then terminated by adding 0.5% SDS, 0.25 µg/ml of bromophenol blue, and 15% glycerol. The samples were electrophoresed in a horizontal 1% agarose gel in Tris-acetate/EDTA buffer (40

mM Tris-acetate, 2 mM EDTA [pH 8]) at 1.5 V/cm for 2-3 h at room temperature. DMSO concentrations in each reaction were maintained at 0.5% by adding serially diluted drug stocks so as not to produce solvent-mediated inhibition of topo I or II activity. The gels were stained with ethidium bromide (5 μg/ml), de-stained in water, and photographed under UV light. The relaxation percentage was measured using a laser microdensitometer (2202 Ultrascan; LKB, Bromma, Stockholm, Sweden) to analyze negative photographs of supercoiled monomer DNA band fluorescence after ethidium bromide staining, and the area under the peak was calculated [28, 29].

**5.11.4. Cell cycle analysis.**

Huh-7 cells were treated with DMSO, **10a** at different concentrations (1.0, 5.0, 10.0 μM) for 24 h. Cells were harvested, rinsed in PBS, resuspended, fixed in 70% ethanol, and stored at -20°C in fixation buffer until ready for analysis. The pellets were suspended in 1 mL of propidium iodide (PI) solution containing 20 μg/μL of PI, 0.2mg/mL RNase, and 0.1% (v /v) Triton X-100. Cell samples were incubated at room temperature in the dark for at least 30 min and analyzed by a flow cytometer (Coulter Epics). Data recording was made using Epics software and cell cycle data were analyzed using Multicycle software (coulter).

**5.11.5. Immunoblot analysis.**

After treatment of compound **10a**, cells were collected and washed twice with cold PBS and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and kept on ice for 30 min. The lysates were centrifuged at 12,000g at 4 $^{o}$C for 20 min and the supernatants were stored at -70$^{o}$C. The protein concentration was determined by the Bradford method. 20 μg protein were separated by 10% SDS-PAGE and transferred onto a PVDF membrane using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol [v/v]). After blocking with 5% non-fat dried milk, the membrane was incubated for 2 h with primary antibodies, followed by 30 min with secondary antibodies in milk containing Tris-buffered saline (TBS)

and 0.5% Tween. The membrane was then exposed to X-ray film. Protein bands were detected using the enhanced chemiluminescence blotting detection system (Amersham, USA).

**5.11.6. Zebrafish xenograft assay**

The zebrafish xenograft assay was used for validating the *in vivo* anti-liver cancer effect of **10a.** The procedure was performed according to a previous study with minor modifications [30]. In brief, the 48 hour post-fertilization (hpf) zebrafish embryos were anesthetized with 0.01%. About 100 cells of Huh-7 were transplanted in to the yolk sac of embryo. Afterwards, embryos were incubated in water with different concentrations of **10a** for 24 and 48 hour post-injection (hpi) respectively. The images of embryos were captured by the fluorescence microscope (Nikon Eclipse TE2000-U, Tokyo, Japan).

**Acknowledgements**

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**List of Figure Captions:**

**Figure 1.** Structures of indeno[1,2-*c*]quinolinone derivatives **1-3**, ellipticine, batracylin, and target compounds.

**Figure 2.** Inhibition of human topoisomerase I (topo I) relaxation activities of indeno[1,2-*b*]quinoxalin-11-one derivatives (A) 100 μM and (B) 10 μM. Lane 1: supercoiled pBR322 DNA alone; lane 2 same as lane 1 but DNA was added after preincubation of topo I with reaction buffer and with 0.5 % DMSO, respectively, for 30 min at 37 ◦C; lane 3: supercoiled DNA was incubated with topo I and compound **2 (**10 μM); remaining lanes: supercoiled DNA was incubated with topo I and indeno[1,2-*b*]quinoxalin-11-one derivatives **(**100 or 10 μM). Inhibition of human topoisomerase II (topo II) relaxation activities of indeno[1,2-*b*]quinoxalin-11-one derivatives (C) 100 μM and (D) 10 μM.. Lane 1**:** supercoiled pBR322DNAalone; lane 2 same as lane 1 but DNA was added after preincubation of topo II with reaction buffer and with 0.5% DMSO, respectively, for 30min at 37 ◦C; lane 3**:** supercoiled DNA was incubated with topo II and compound **2** (10 μM); remaining lanes: supercoiled DNA was incubated with topo II and indeno[1,2-*b*]quinoxalin-11-one derivatives **(**100 or 10 μM).

**Figure 3.** Flow cytometric analysis of Huh-7 cells. Cells were treated with DMSO (A), 1.0 μM (B), 5.0 μM (C) or 10.0 μM (D) of **10a** in Huh-7 cells; 24 h later the cells were harvested, fixed, and stained with propidium iodide as described in Experimental Section prior to analysis by flow cytometry. The percentage of cells in each cell cycle phase was quantified (Table 2).

**Figure 4**. Induction of morphological change in Huh-7 cells. Cells were treated with DMSO or compound **10a** (1.0-10.0 μM) for 24 h at 37 °C and photographed under a microscope.

**Figure 5.** Immunoblot analysis for the levels of cell cycle regulatory proteins. Cells were treated with DMSO or 1, 5, and 10 μM of **10a** for the 24 hr. Total cell lysates were prepared and 50 μg protein was subjected to SDS–PAGE followed by Western blot analysis. Each antigenic protein was detected by using the respective antibodies against Cyclin A, Cyclin B1,

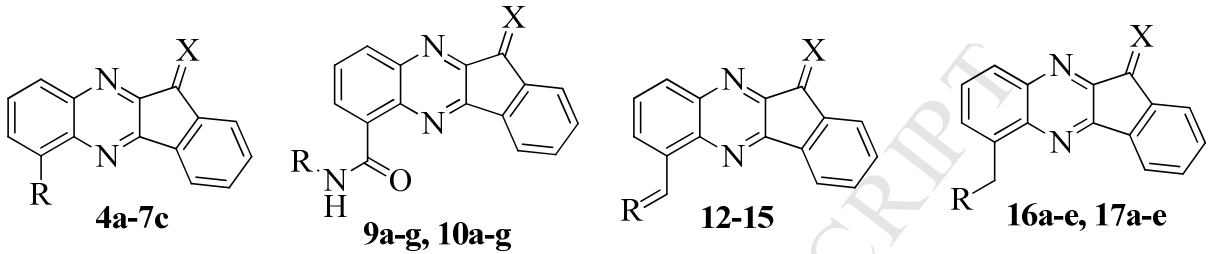
Cyclin D1 or β-Actin. Intensities of the immunoreactive bands were quantified by densitometric scanning.

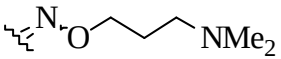
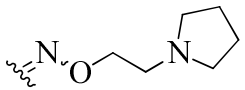
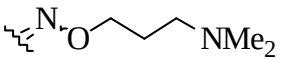
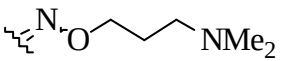
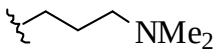
**Figure 6.** Effects of **10a** on the expression of procapase-3, -7, Bax, Bcl-2, and PARP in Huh-7 cells. Exponentially growing Huh-7 cells were treated with the indicated concentrations of **10a** for 24 h. Cell lysates were prepared and protein levels of procapase-3, -7, Bax, Bcl-2, Bax, and PARP were determined by Western blotting analysis. β-Actin was used to confirm equal protein loading.

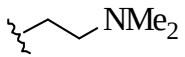
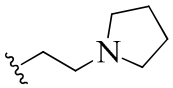
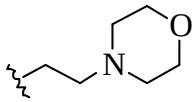
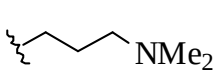
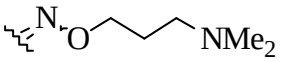
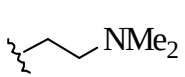
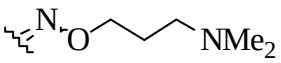
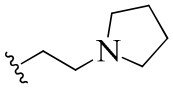
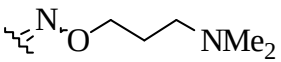
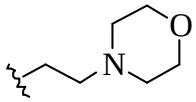
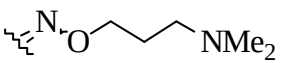
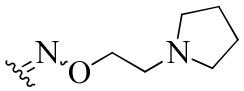
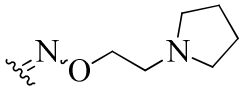
**Figure 7.** Effect of **10a** on the expression levels of, p-Src, p-Akt-1, p-Akt-2 and Akt. Cells were treated with DMSO or 1, 5, and 10 μM of **10a** for the 24 hr, after which whole cell extracts were prepared, and 50 μg proteins of these extracts were resolved by SDS–PAGE, and then immunoblotted with specific antibodies. β-Actin was used as an equal loading control.

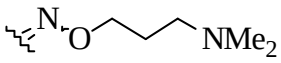
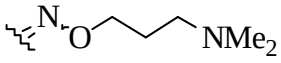
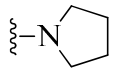
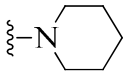
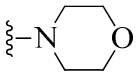
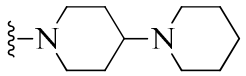
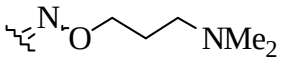
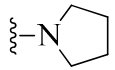
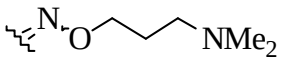
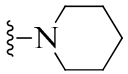
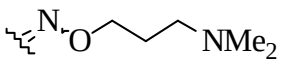
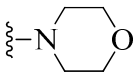
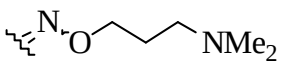
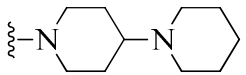
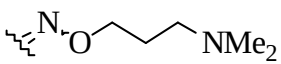
**Figure 8.** (A) Determination of the human tumor size in the zebrafish xenograft assay. The intensity of red fluorescence is proportional to the xenograft tumor size (B) From 0.1 to 2.0 μg/ml **10a** treatments no significant change in the survival rate of the zebrafish transplanted with Huh-7 tumor cells. n = 20 embryos for each group.

**Table 1**. Antiproliferative activity and DNA binding affinity of indeno[1,2-*b*]quinoxalin-11-one derivatives ($IC_{50}$, μM)




| compds | R | X | DNA binding affinity | $IC_{50}$ (μM) / selectivity index (SI[a]) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | MDA-MB231 | H1299 | PC-3 | Huh-7 | MRC-5 |
| **4a** | H | O | - | > 10 | > 10 | > 10 | > 10 | 85.32 ± 2.85 |
| **4b** | Me | O | - | > 10 | > 10 | > 10 | > 10 | 87.08 ± 1.87 |
| **4c** | COOH | O | - | > 10 | > 10 | > 10 | > 10 | 71.23 ± 3.14 |
| **5a** | H | NOH | - | > 10 | > 10 | > 10 | > 10 | 51.12 ± 1.47 |
| **6a** | H |  | - | 4.92 ± 0.03 (3.59) | 7.03 ± 0.07 (2.51) | 8.51 ± 0.11 (2.07) | 6.46 ± 0.06 (2.73) | 17.65 ± 2.17 |
| **7a** | H |  | - | 7.01 ± 0.05 (2.83) | 7.19 ± 0.24 (2.76) | 8.63 ± 0.49 (2.30) | 5.38 ± 0.34 (3.69) | 19.87 ± 1.35 |
| **7b** | Me |  | - | 6.84 ± 0.08 (1.13) | 7.26 ± 0.43 (1.08) | 7.01 ± 0.19 (1.12) | 5.88 ± 0.06 (1.34) | 7.86 ± 0.34 |
| **7c** | COOH |  | - | > 10 | > 10 | > 10 | > 10 | 15.14 ± 2.87 |
| **9a** |  | O | - | 7.04 ± 0.12 | 7.82 ± 1.71 | 10.17 ± 1.01 | 7.70 ± 0.31 | 9.07 ± 0.55 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | (1.29) | (1.16) | (0.89) | (1.18) | |
| **9b** |  | O | - | 7.29 ± 0.04 (1.23) | 8.75 ± 0.07 (1.03) | 10.97 ± 1.75 (0.82) | 7.64 ± 0.39 (1.18) | 8.98 ± 0.22 |
| **9c** |  | O | - | 6.55 ± 0.21 (1.22) | 11.24 ± 1.82 (0.71) | 11.98 ± 1.12 (0.67) | 8.51 ± 0.45 (0.94) | 7.99 ± 0.11 |
| **9d** |  | O | - | > 10 | > 10 | > 10 | > 10 | 88.61 ± 3.64 |
| **10a** |  |  | + | 0.87 ± 0.11 (36.22) | 5.78 ± 0.43 (5.45) | 0.82 ± 0.29 (38.43) | 0.64 ± 0.09 (49.23) | 31.51 ± 1.46 |
| **10b** |  |  | + | 0.67 ± 0.07 (13.43) | 6.88 ± 0.51 (1.31) | 0.89 ± 0.39 (10.11) | 0.83 ± 0.04 (10.84) | 9.00 ± 0.30 |
| **10c** |  |  | + | 0.45 ± 0.08 (17.53) | 5.69 ± 1.47 (1.39) | 0.68 ± 0.23 (11.60) | 0.82 ± 0.07 (9.62) | 7.89 ± 0.37 |
| **10d** |  |  | + | 0.99 ± 0.14 (11.63) | 9.69 ± 0.12 (1.19) | 9.78 ± 0.26 (1.18) | 5.94 ± 0.04 (1.94) | 11.52 ± 1.87 |
| **12** | O | O | - | > 10 | > 10 | > 10 | > 10 | 78.91 ± 2.51 |
| **13** | NOH | NOH | - | 10.76 ± 0.55 (0.61) | 8.13 ± 0.98 (0.80) | 9.45 ± 2.25 (0.69) | 8.61 ± 0.15 (0.76) | 6.54 ± 0.08 |
| **14** |  |  | + | 1.01 ± 0.18 (0.90) | 6.02 ± 0.13 (0.15) | 5.17 ± 0.06 (0.18) | 0.78 ± 0.05 (1.17) | 0.91 ± 0.06 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **15** |  |  | - | 7.03 ± 0.67 (0.93) | 5.10 ± 0.18 (1.29) | 6.27 ± 0.07 (1.05) | 7.19 ± 0.05 (0.91) | 6.56 ± 0.09 |
| **16a** |  | O | - | > 10 | > 10 | > 10 | > 10 | 77.81 ± 1.92 |
| **16b** |  | O | - | > 10 | > 10 | > 10 | 6.98 ± 0.11 (1.40) | 9.80 ± 0.28 |
| **16c** |  | O | - | > 10 | > 10 | > 10 | > 10 | 25.87 ± 1.98 |
| **16d** |  | O | - | > 10 | > 10 | > 10 | > 10 | 95.68 ± 2.57 |
| **16e** |  | O | - | > 10 | > 10 | > 10 | > 10 | 89.71 ± 3.14 |
| **17a** |  |  | + | 0.90 ± 0.06 (0.98) | 0.81 ± 0.08 (1.09) | 0.78 ± 0.05 (1.13) | 0.75 ± 0.05 (1.17) | 0.88 ± 0.14 |
| **17b** |  |  | + | 0.68 ± 0.09 (1.32) | 0.92 ± 0.12 (0.98) | 0.78 ± 0.07 (1.15) | 0.32 ± 0.06 (2.81) | 0.90 ± 0.08 |
| **17c** |  |  | + | 0.66 ± 0.06 (1.35) | 0.83 ± 0.13 (1.07) | 0.91 ± 0.05 (0.98) | 0.63 ± 0.07 (1.41) | 0.89 ± 0.13 |
| **17d** |  |  | - | 9.97 ± 2.19 (7.37) | 6.93 ± 0.24 (10.60) | 9.92 ± 1.61 (7.41) | 6.78 ± 0.52 (10.84) | 73.49 ± 2.15 |
| **17e** |  |  | - | 0.99 ± 0.08 (3.53) | 5.14 ± 0.17 (0.68) | 6.12 ± 0.21 (0.57) | 0.82 ± 0.03 (4.26) | 3.49 ± 0.35 |
| | Topotecan | | ND[b] | < 0.1 | 6.02 ± 0.20 | 9.35 ± 1.24 | 8.61 ± 1.14 | 4.25 ± 0.81 |

| | | | | |
|---|---|---|---|---|
| | (> 42.5) | (0.71) | (0.45) | (0.49) |

[a]SI: Selectivity index = ($IC_{50}$ of MRC-5) / ($IC_{50}$ of cancer cell line).

[b]ND: Not determined

**Table 2.** Topoisomerases (topo I and topo II) inhibitory activities[a] of indeno[1,2-*b*]quinoxalin-11-one derivatives.

| Compd. | Top I inhibition at 100 μM | Top I inhibition at 10 μM | Top II inhibition at 100 μM | Top II inhibition at 10 μM |
|---|---|---|---|---|
| **2** | +++ | ++ | +++ | ++ |
| **4a** | ND[b] | ND | ND | ND |
| **4b** | ND | ND | ND | ND |
| **4c** | ND | ND | ND | ND |
| **5a** | - | ND | - | ND |
| **6a** | ++ | + | +++ | - |
| **7a** | ++ | + | +++ | - |
| **7b** | ++ | + | +++ | - |
| **7c** | - | ND | - | ND |
| **9a** | ++ | - | +++ | - |
| **9b** | ++ | + | +++ | - |
| **9c** | ++ | + | +++ | - |
| **9d** | - | ND | - | ND |
| **10a** | ++ | ++ | ++ | ++ |
| **10b** | ++ | ++ | ++ | ++ |
| **10c** | ++ | ++ | ++ | ++ |
| **10d** | ++ | ++ | ++ | - |
| **12** | - | ND | - | ND |
| **13** | - | ND | - | ND |
| **14** | ++ | ++ | ++ | ++ |
| **15** | ++ | ++ | ++ | ++ |
| **16a** | ++ | - | - | ND |
| **16b** | ++ | - | - | ND |
| **16c** | ++ | - | - | ND |
| **16d** | - | ND | - | ND |
| **16e** | - | ND | - | ND |
| **17a** | ++ | ++ | + | - |
| **17b** | ++ | ++ | + | - |
| **17c** | ++ | ++ | + | - |
| **17d** | ++ | - | + | - |
| **17e** | ++ | ++ | + | - |

[a]Topo I and topo II inhibitory activities of compounds were expressed semi-quantitatively as follows: -: very weak activity; +: weak activity; ++: moderate activity; +++: strong activity.
[b]ND: Not determined

**Table 3.** Effects of **10a** on Huh-7 cell cycle progression.

| Concentration (μM) | Cell cycle distribution (%)[a] | | | |
|---|---|---|---|---|
| | Sub G1 | G1 | S | G2/M |
| DMSO | 1.3 ± 0.7 | 62.1 ± 1.4 | 11.4 ± 2.4 | 25.2 ± 3.5 |
| 1.0 | 3.8 ± 1.1 | 55.3 ± 2.9 | 21.6 ± 2.5 | 19.3 ± 1.7 |
| 5.0 | 11.5 ± 1.9 | 35.1 ± 3.7 | 30.5 ± 2.3 | 22.9 ± 4.1 |
| 10.0 | 23.4 ± 3.7 | 30.4 ± 2.1 | 38.4 ± 3.4 | 7.8 ± 1.9 |

[a]Values representative mean ± SD from three experiments.

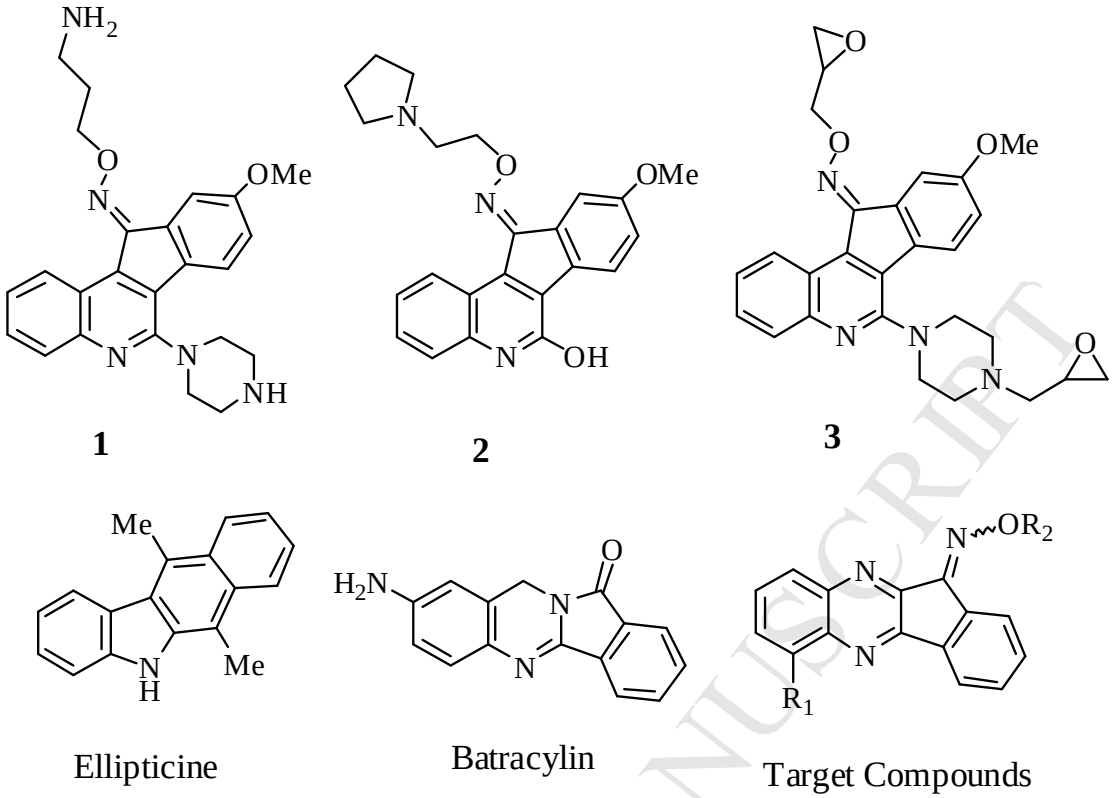
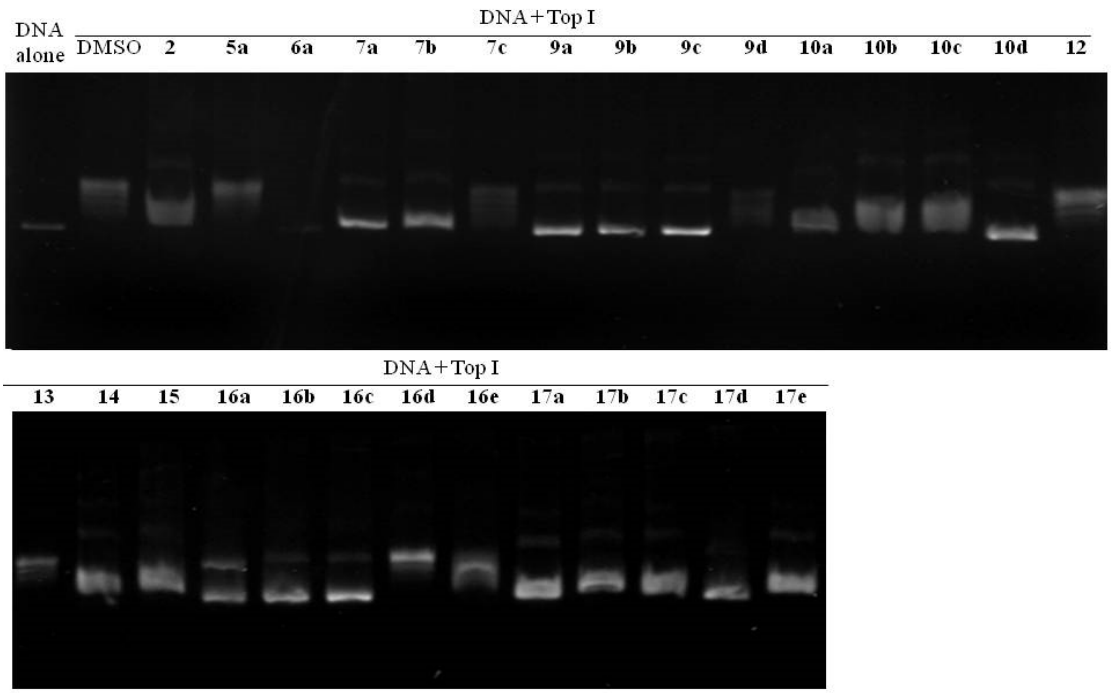
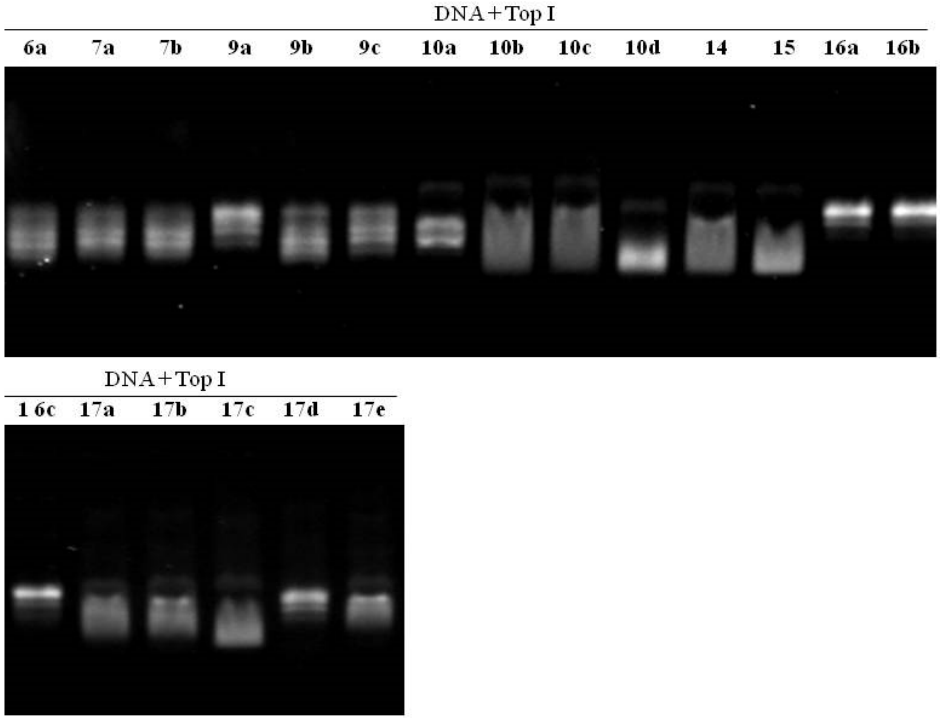



**Figure 1.**

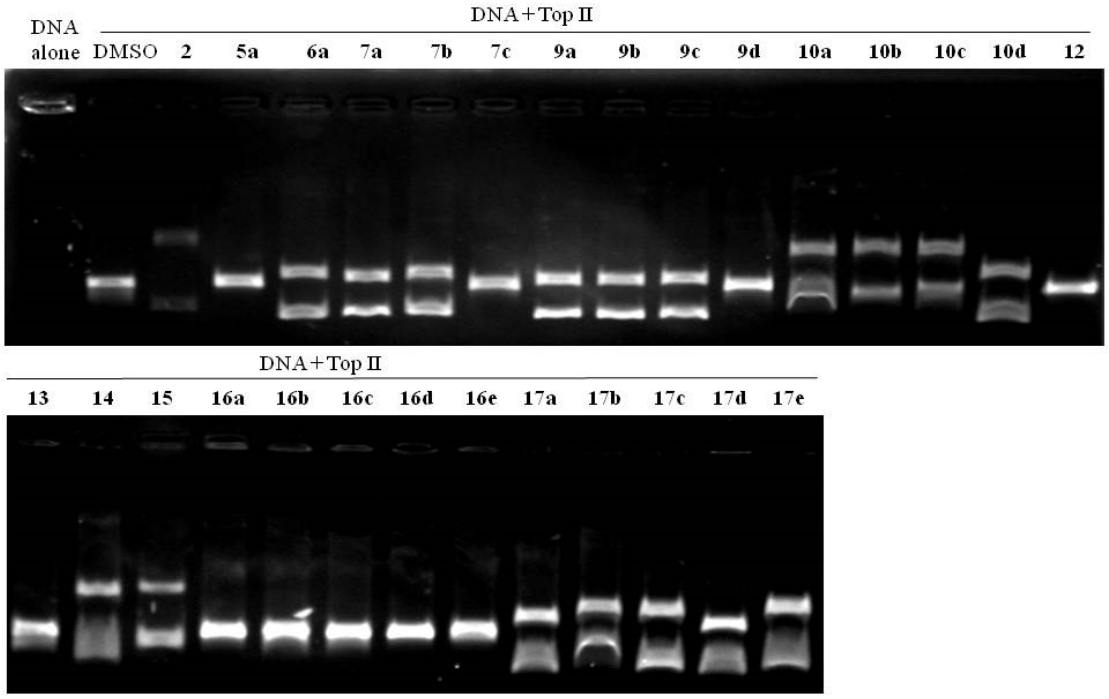
**(A)**


DNA alone
DNA+Top I
DMSO 2 5a 6a 7a 7b 7c 9a 9b 9c 9d 10a 10b 10c 10d 12
DNA+Top I
13 14 15 16a 16b 16c 16d 16e 17a 17b 17c 17d 17e

**(B)**


DNA+Top I
6a 7a 7b 9a 9b 9c 10a 10b 10c 10d 14 15 16a 16b
DNA+Top I
16c 17a 17b 17c 17d 17e

**(C)**


DNA alone
DNA+Top II
DMSO
2
5a
6a
7a
7b
7c
9a
9b
9c
9d
10a
10b
10c
10d
12
DNA+Top II
13
14
15
16a
16b
16c
16d
16e
17a
17b
17c
17d
17e

**(D)**

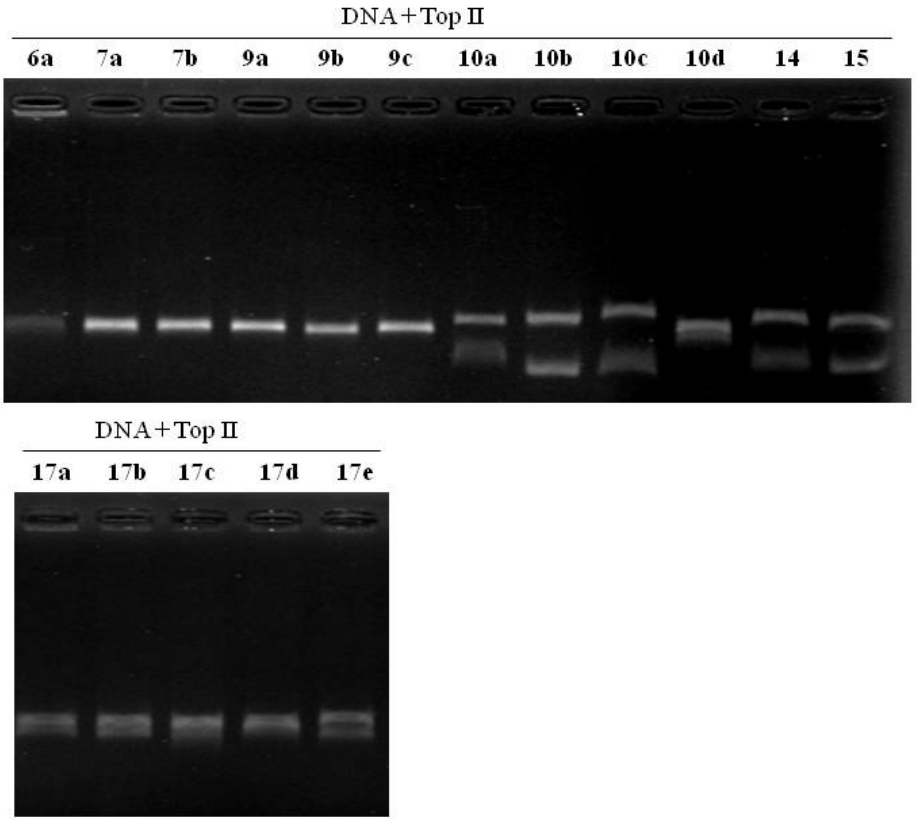

DNA+Top II
6a
7a
7b
9a
9b
9c
10a
10b
10c
10d
14
15
DNA+Top II
17a
17b
17c
17d
17e

**Figure 2.**

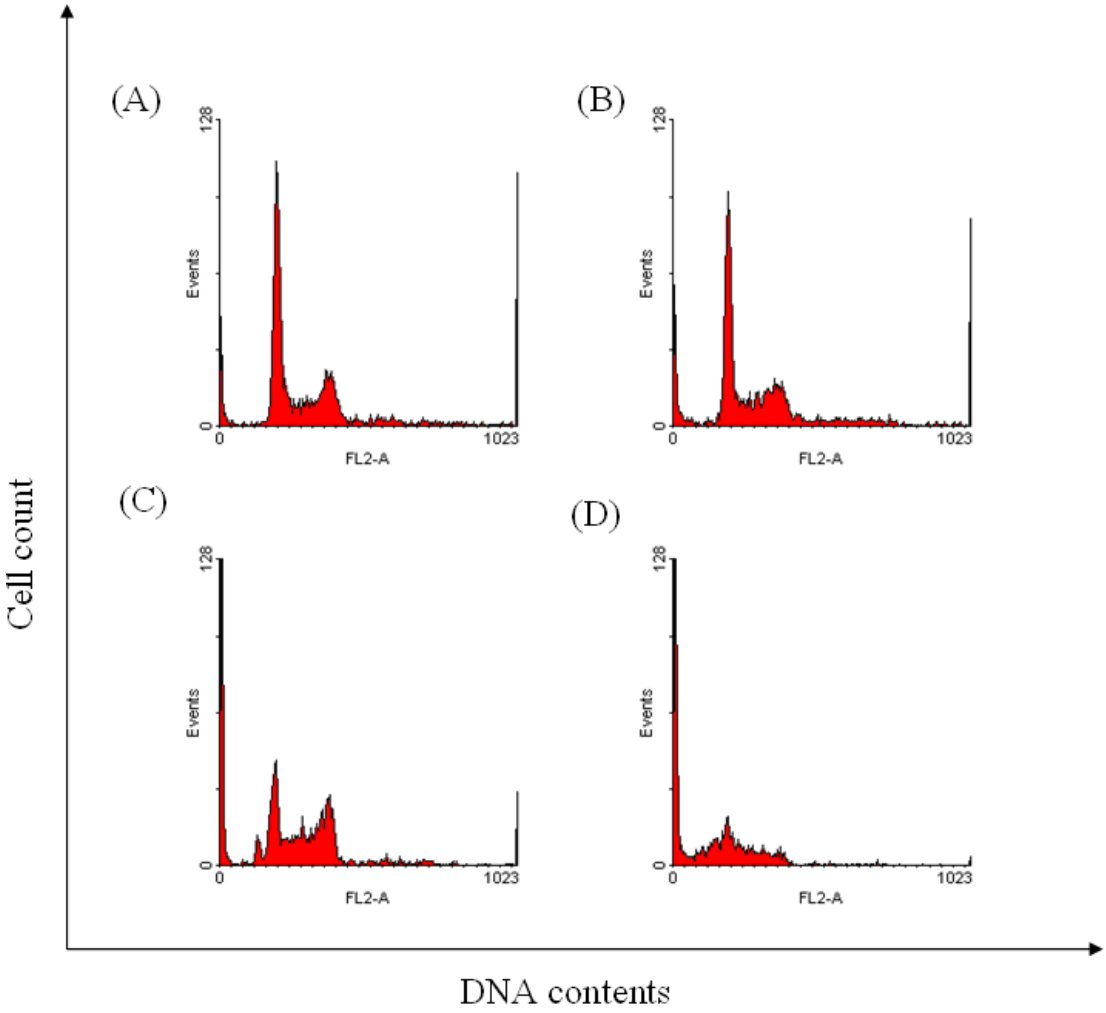
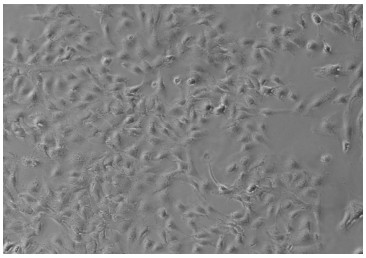
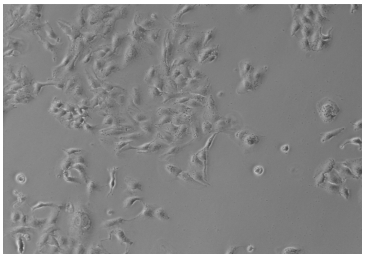



**Figure 3.**

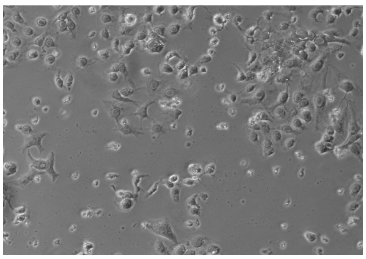
DMSO



1.0 μM



5.0 μM



10.0 μM

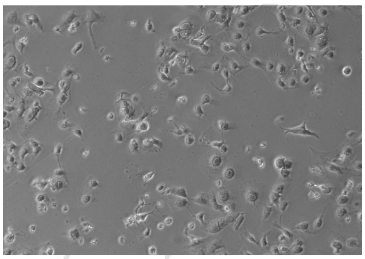


**Figure 4**.

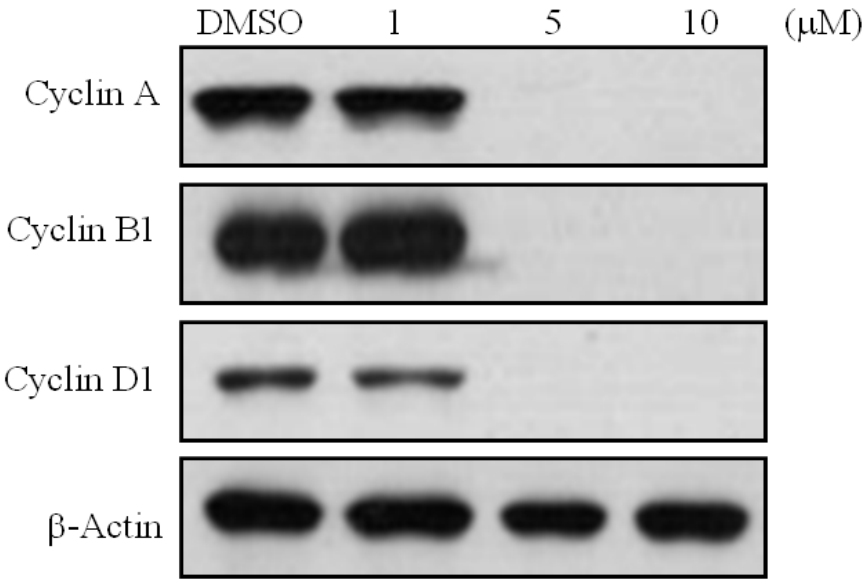



**Figure 5.**

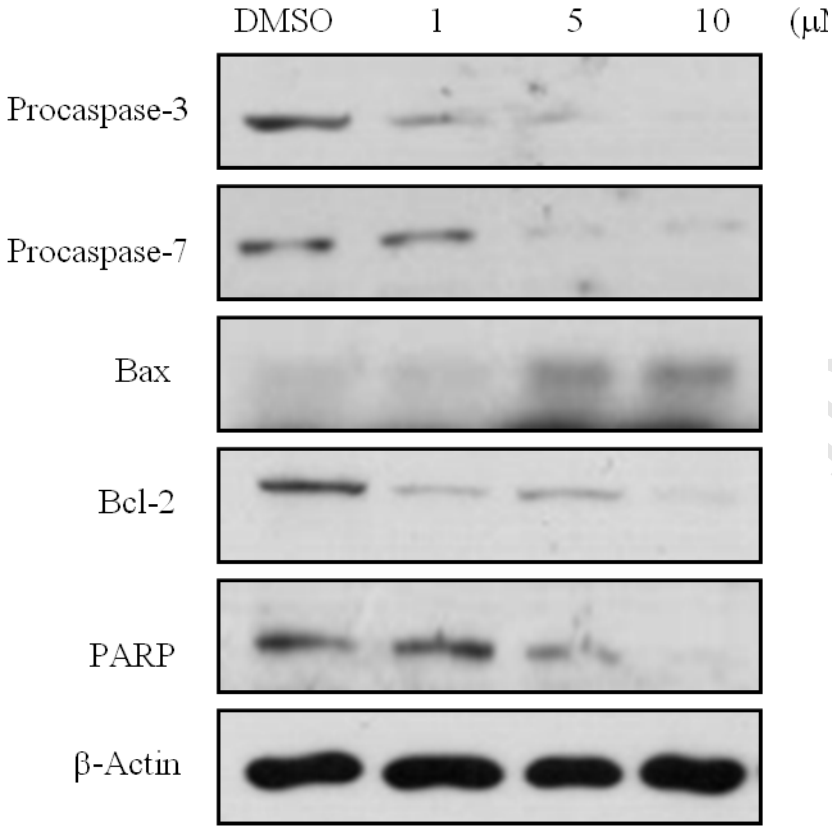



**Figure 6.**

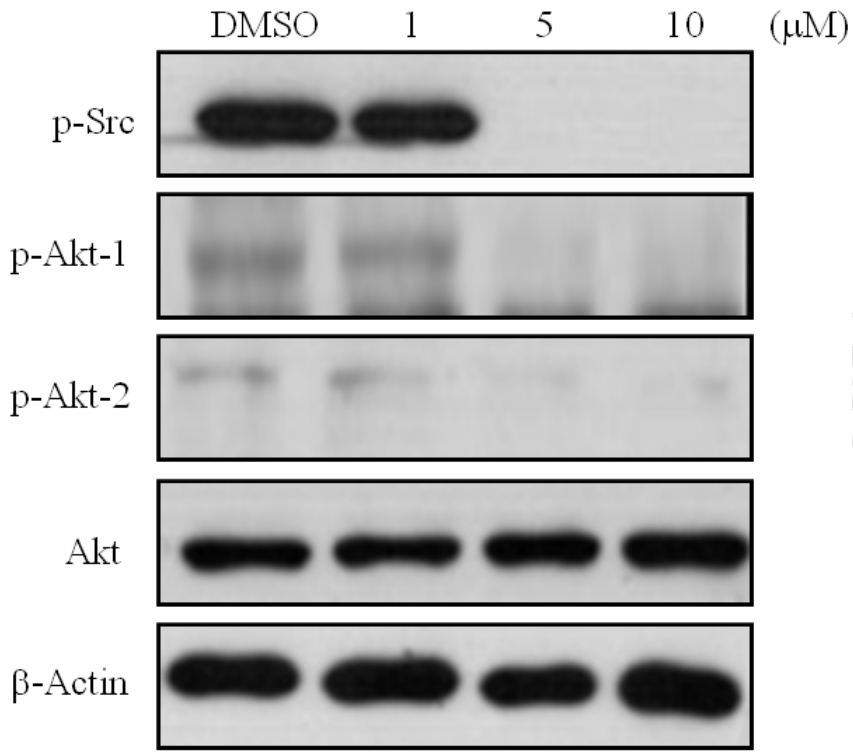
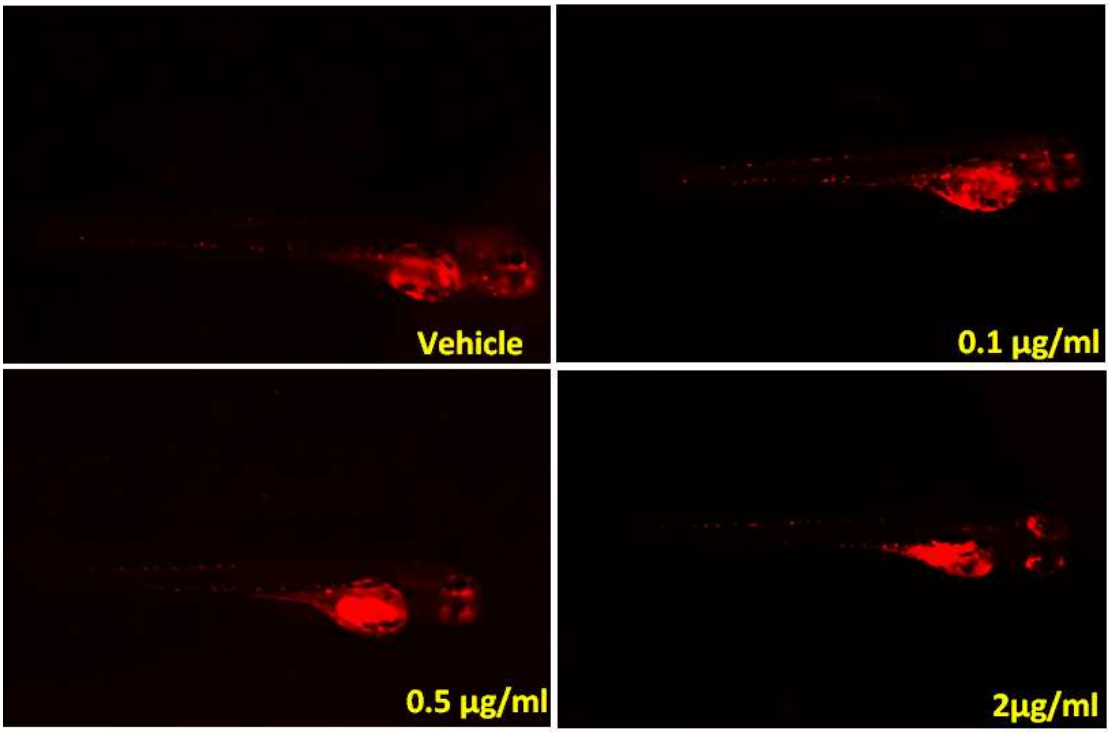



**Figure 7.**

**(A)**


Vehicle
0.1 µg/ml
0.5 µg/ml
2µg/ml

**(B)**

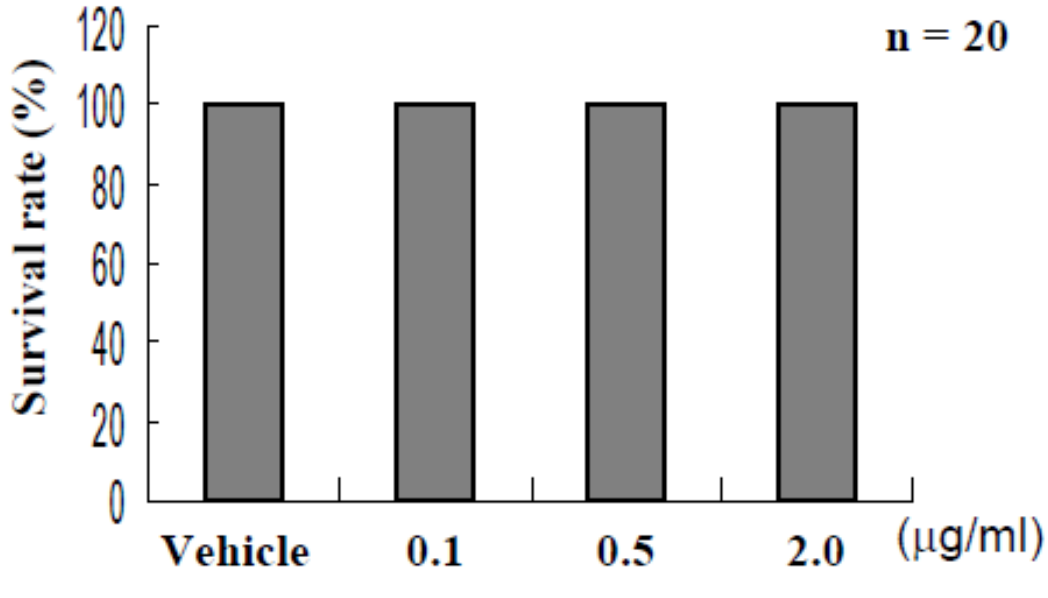
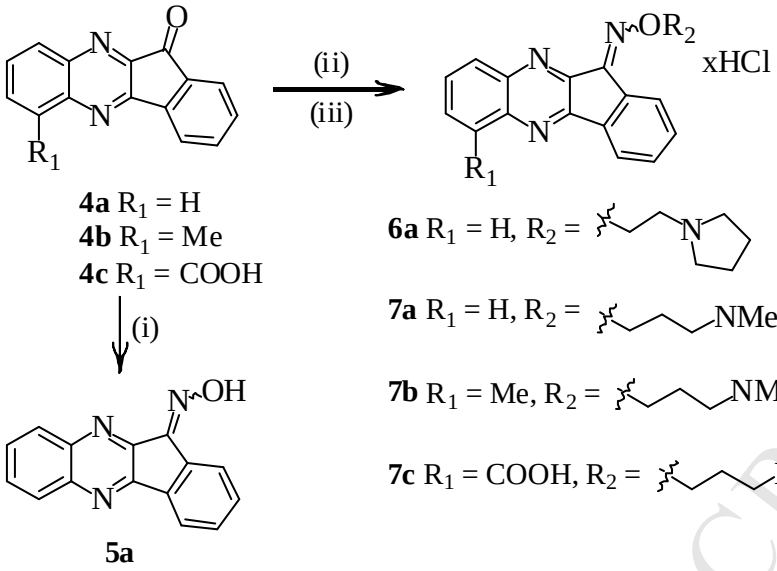
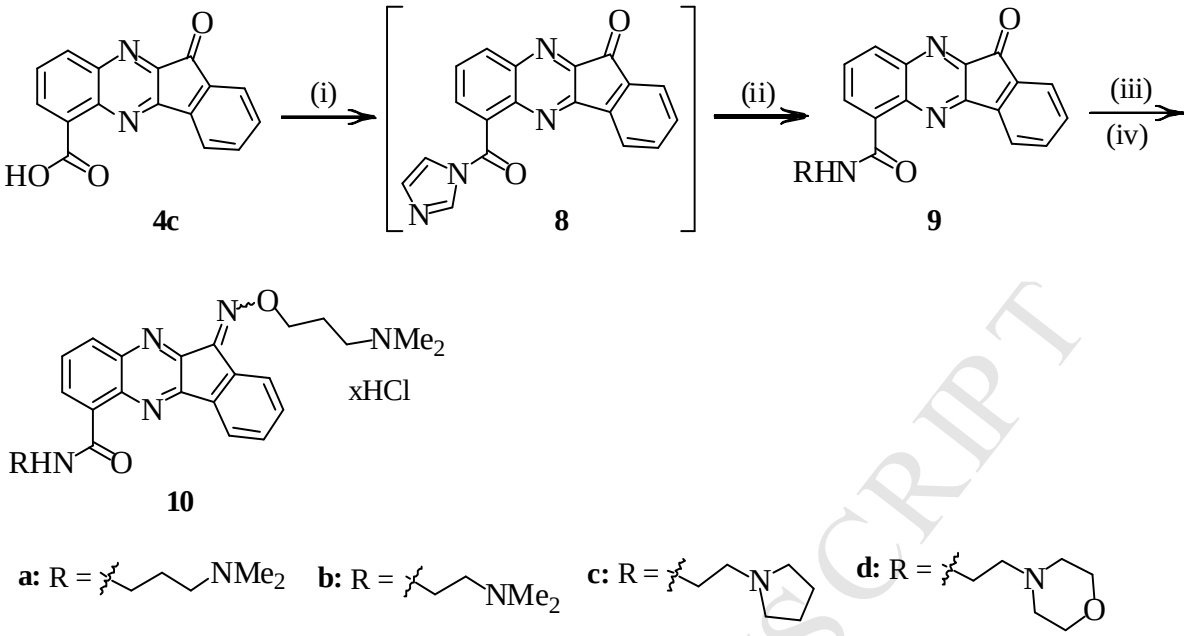

n = 20
Survival rate (%)
120
100
80
60
40
20
0
Vehicle
0.1
0.5
2.0
(µg/ml)

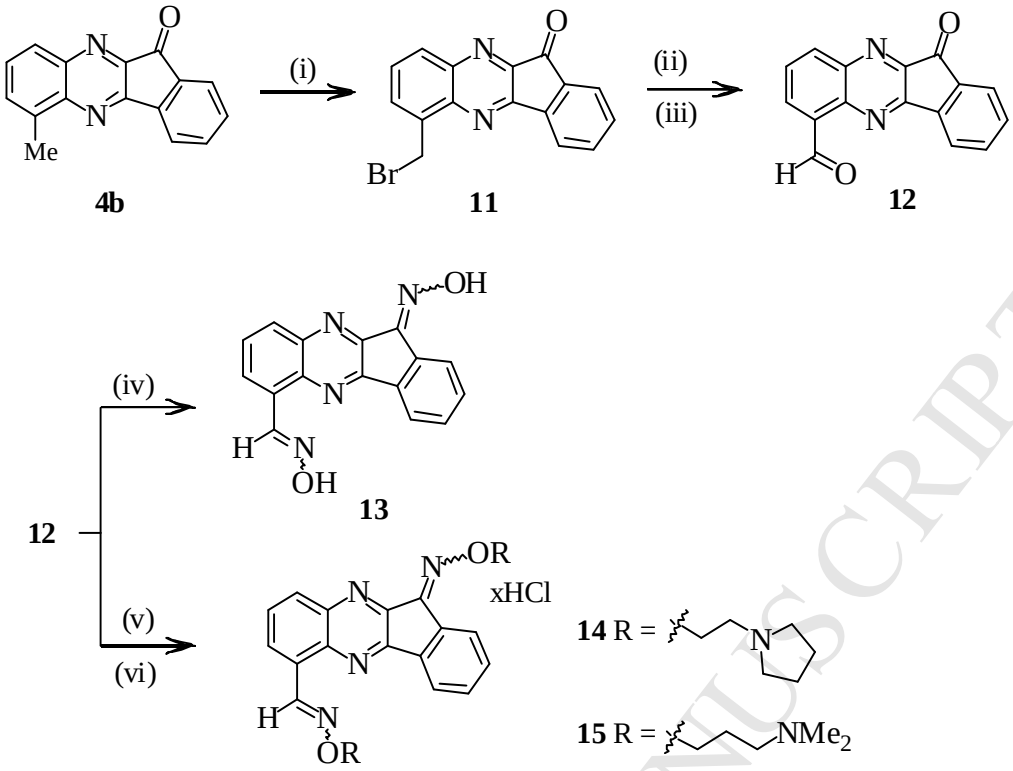
**Figure 8.**



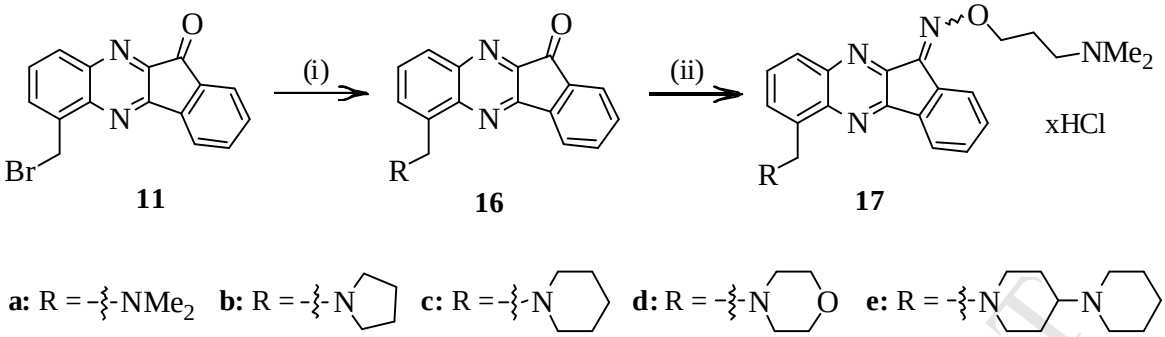

**Scheme 1**: Reagents and conditions: (i) $NH_2OH$, EtOH, reflux, 6 h, (ii) $NH_2OR_2$, EtOH, reflux, 6 h, (iii) 3N HCl, EtOH, rt, 30 min.




**Scheme 2**: Reagents and conditions: (i) CDI, dioxane, reflux, 4 h; (ii) $RNH_2$, $CH_2Cl_2$, rt, 10 min or THF, reflux, 6 h; (iii) $NH_2O(CH_2)_3N(Me)_2$, EtOH, reflux, 6 h, (iv) 3N HCl, EtOH, rt, 30 min.




**Scheme 3**: Reagents and conditions: (i) NBS, BPO, $CCl_4$, reflux, 2 h; (ii) $CaCO_3$, dioxane/$H_2O$ (4/1), reflux, 48 h; (iii) $MnO_2$, $CH_2Cl_2$, rt, 12 h; (iv) $NH_2OH$, EtOH, reflux, 6 h; (v) $NH_2OR$, EtOH, reflux, 6 h; (vi) 3N HCl, EtOH, rt, 30 min.




**11** **16** **17**

**a:** R = -NMe$_2$ **b:** R =  **c:** R =  **d:** R =  **e:** R = 

**Scheme 4**: Reagents and conditions: (i) secondary amines, $CH_2Cl_2$, rt, 10 min, or THF, rt, 10 min; (ii) $NH_2O(CH_2)_3N(Me)_2$, EtOH, reflux, 6 h

**Highlights**

- Novel series of indeno[1,2-*b*]quinoxaline derivatives were synthesized.
- Most indeno[1,2-*b*]quinoxalines exhibited potent cytotoxicity against cancer cells.
- Compound **10a** induced *S*-phase arrest and apoptosis in Huh-7 cells.
- Compound **10a** inhibited tumor growth in the Huh-7 tumor zebrafish xenograft model.