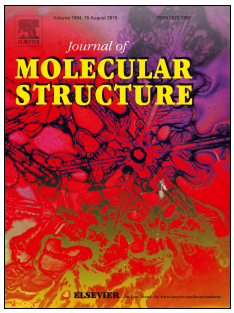

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Saiprasad N. Nunewar: Mechanistic studies, Viscosity studies-

Naveen Kotla, Apoorva Dixit: Synthesis and characterization.

Jaya Lakshmi Uppu: Viscosity studies, Nanodrop method.

Venkatesh Pooladanda, Chandraiah Godugu: MTT assay

Neelima D. Tangellamudi: Concept, Writing, Reviewing and Editing

Synthesis of 1-(Indol-2-yl)-Phenoxazine Hybrids from Quinacetophenone Precursors and their Biological Evaluation as DNA Intercalating Agents

Saiprasad N. Nunewar,[a] Naveen Kotla,[a] Jaya Lakshmi Uppu,[b] Apoorva Dixit,[a] Venkatesh Pooladanda,[b] Chandraiah Godugu,[b] Neelima D. Tangellamudi*, [a]

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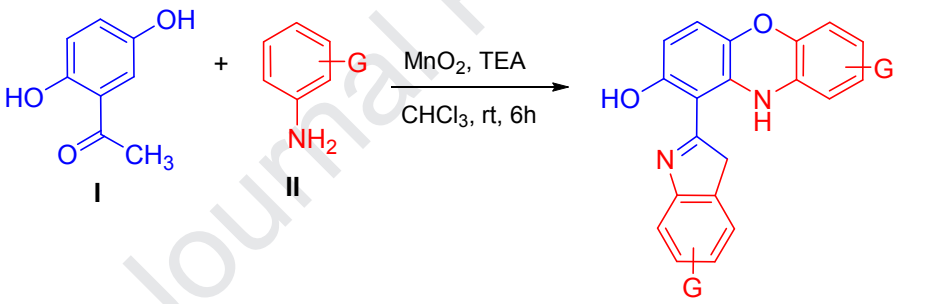

1-(Indol-2-yl) Phenoxazines
40-62% 15 examples

G= H, 4-Cl, 4-F, 4-CH_3, 2,3-di-CH_3, 2,4-di-CH_3, 2,5-di-CH_3, 4-^{i}Pr 2-MeO, 4-OMe, 3,4-di-OMe,3,4,5-tri-OMe

- **Short reaction time**
- **New frameworks**
- **Anti-cancer activity**
- **Ability to intercalate DNA**

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Abstract

An operationally simple reaction between quinacetophenone and aniline derivatives in the presence of triethylamine at room temperature afforded 1-(indol-2-yl)-phenoxazine hybrids in good yields. This unique transformation proceeds *via* sequential aza-Michael attack and imine formation to yield 1-(indol-2-yl)-phenoxazine hybrids. The most plausible reaction mechanism was established based on the mechanistic studies carried out with Mass spectrometry.The synthesized molecules were subjected to *in-vitro*anti-proliferative evaluation on various cancer cell lines by MTT assay where compound **8**was found to display signifiacnt IC_{50} value of $\mathbf{3.71 \pm 0.57\ \mu M}$ on **A-549** cell line. The ability of compound **8** to intercalate DNA was also confirmed by DNA Nanodrop method and viscosity experimental studies.

Keywords:*Quinacetophenone, Aza-Michael attack, Phenoxazine hybrids, DNA intercalation.*

Introduction

Quinacetophenone (2,5-dihydroxyacetophenone) is an easily accessible precursor for the syntheses of different pharmacologically active compounds.[1] Quinacetophenone is an important scaffoldfrom the viewpoints of both synthesis and chemical reactivity that is inherent in the hydroxyl, methyl ketone, carbonyl and aromatic moieties. Thus quinacetophenone has been used as a precursor to afford privileged motifs of various kinds viz., chalcones, chromones, flavonoids, coumarins, aurones, phenazines and azoles.[2]

In presence of various oxidants, quinacetophenone is oxidized to give 2-acetyl-[1,4]-benzoquinone **A,**[3a,b] which is most prominently, attacked by nucleophiles selectively at C-6 providing 6-substituted quinacetophenone, driven by the resonance stabilization associated with the intermediate **I** as shown in **Figure.1**.[4] The 1-acetyl group is also known for its high reactivity which could be attributed to proton donation to the carbonyl group from the adjacent 2-hydroxy group and resonance of the carbonyl group with the aromatic ring .[5,6]

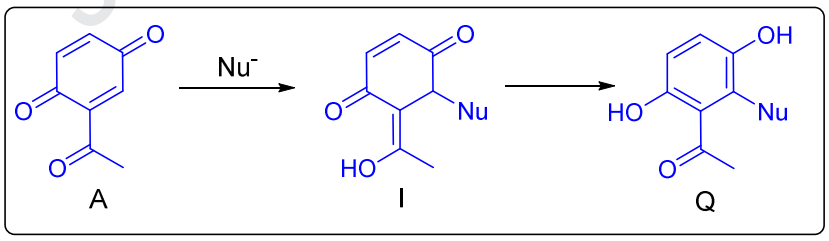


Figure 1. 2-acetyl 1,4- benzoquinone (A); Intermediate **(I**) and formation of 6-substituted quinacetophenone (Q) by rearomatization

We have earlier reported the synthesis of benzoxazines[7a] and benzoxazoles[7b]from 2,5-dihydroxy benzaldehyde by a novel one pot synthesis using manganese dioxide an oxidant. Further, we have recently reported a methodology for a facile synthesis of phenoxazines from

quinacetophenone precursor and anilines in the presence of an oxidizing agent and absence of any additive.[7c]Benzoxazines/phenoxazines have been studied intensively as important heterocyclic system for the synthesis of herbicides, fungicides and therapeutically usable drugs.[8a,b] Several benzoxazine derivatives are currently in the development phase as potential new drugs.[11c]Indole is a prominent constituent of flower perfumes, pharmacologically active alkaloids, therapeutics, drug candidates and hormones.[9a] Some naturally occurring indole alkaloids including vincristine, vinblastine and vindesine for anti-tumor activity have gained FDA approval.[9b,10] In view of the potential pharmacological effects of indoles and phenoxazines and recognizing the enhanced synergistic effects that the pharmacophore hybrids offer,[11,12,13]we presumed that hybrids containing indole and phenoxazine in a single heteroaromatic framework might offer potential anti-proliferative effect. Further, our goal was to evaluate their ability as DNA intercalators owing to their typical structural features

DNA intercalators are molecules capable of fitting between nucleic acid base pairs in a reversible action. The intercalation process [14] involves the transfer of the intercalating molecule from an aqueous environment to the hydrophobic space between two adjacent DNA base pairs. DNA undergoes conformational changes involving an increase in the vertical separation in order to accommodate the ligand between the base pairs. Once the ligand gets sandwiched between the DNA base pairs, the stability of the complex is optimized by a number of non-covalent interactions, ionic interactions and hydrogen bonding.[15]This intercalation prevents DNA replication, leading to possible cell death and anti-cancer action in rapidly growing cancer cells.

The characteristic features of the synthesized compounds like fused ring structure of phenoxazine chromophore, presence of two basic groups that can be protonated easily and the relatively planar geometry of the compounds are the typical features of DNA intercalating agents. These features impart DNA intercalating ability to these chemical structures, as

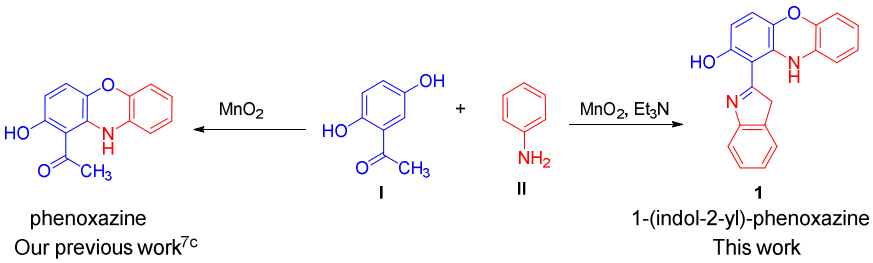
cationic species are more efficient DNA intercalators because they interact better with the negatively charged DNA sugar–phosphate backbone under physiological conditions.[15] The planar geometry allows the DNA to accommodate the molecule between the base pairs. Thus we embarked on a journey to synthesize these 1-(indol-2-yl)-phenoxazine hybrids from an operationally simple reaction between quinacetophenone and aniline derivatives and further evaluate their ability to intercalate DNA.

Results and discussion

Optimization conditions

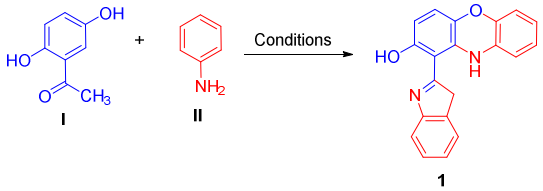
As a sequel to the synthesis of phenoxazines,[7c] we wished to arrive at phenoxazine-indole hybrids by extending the same methodology by addition of an additive. In this context, we reasoned that triethylamine might activate the carbonyl for imine formation and a probable cyclization to indoles to afford our much desired hybrids.

Thus triethylamine was added as an additive to investigate its effect on activating the carbonyl group to validate our reasoning. As expected, a mere addition of 0.5 equiv. of triethylamine to the reaction mixture containing quinacetophenone, manganese dioxide and aniline in toluene, led to the formation of 1-(indol-2-yl)-phenoxazine hybrid. (**Scheme. 1)**



Scheme 1:Synthesis of phenoxazines and 1-(indol-2-yl)-phenoxazine from quinacetophenone

Table 1. Optimization of reaction conditions[a]



S.No	Oxidant	Additive	Solvent	Temp.	Yield[b]
1	MnO_2	TEA	Toluene	r.t	22%
2	MnO_2	TEA	Toluene	Reflux	12%
3	MnO_2	TEA	Dioxane	r.t.	27%
4	MnO_2	TEA	DCE	r.t.	45%
5	**MnO_2**	**TEA[c]**	**$CHCl_3$**	**r.t.**	**62%**
6	Ag_2O	TEA	$CHCl_3$	r.t.	30%
7	MnO_2	-	$CHCl_3$	r.t.	30%[d]
8	PIDA	-	CH_2Cl_2	r.t.	15%[d]
9	PIDA	TEA	CH_2Cl_2	r.t.	13%
10	PIDA	TEA	DCE	r.t.	09%
11	$Pb(OAc)_2$	TEA	CH_2Cl_2	r.t.	19%
12	Ag_2O	-	$CHCl_3$	r.t.	10%[d]

[a]Reaction condition: Quinacetophenone (l, 1.0 mmol), Aniline (ll, 2.0 mmol), Oxidant (10.0 mmol), solvent (10 mL), b: The yields were 11% when I and II were taken in 1:1 ratio which increased to 22% when taken in 1:2 ratios, c: 0.5 equivafforded optimum yields, d: Simple phenoxazine was obtained [7c].

Our optimization studies of the reaction started by using **I** and **II** in 1:1 ratioas the starting materials in presence of manganese dioxide as oxidant in toluene, in the presence of 1 equivalent of triethylamine, wherein we obtained 1-(indol-2-yl)-phenoxazine hybrid, **1** in

11% yields (**Table 1**, **entry 1)**. The yields have increased to 22% when **I** and **II** were takenin 1:2 ratios. Thus we continued using **I** and **II** in 1:2 ratios and 0.5 equiv. of triethylamine for further optimization. Increase in the amount of triethylamine had no positive effect on the yields or reaction times. Encouraged by the formation of 1-(indol-2-yl)-phenoxazine hybrids, we have tried other oxidants such as silver (I) oxide, phenyl iodine diacetate (PIDA) and lead acetate in different solvents like toluene, dioxane, dichloroethane and chloroform to improve the yields. Reaction using silver (I) oxide in presence of triethylamine in chloroform showed slight improvement in yields (**Table 1**, **entry 6**). In the absence of triethylamine, PIDA, silver (I) oxide and manganese dioxide afforded simple phenoxazine in 30% yields (**Table 1**, **entries 7, 8 and 12**).[7c] When we performed reaction with PIDA, the product **1** was obtained in 13% and 9% respectively in dichloromethane and dichloroethane respectively (**Table 1**, **entries 9** and **10**). Lead acetate afforded the product **1** in 19% yields (**Table 1**, **entry 11**).

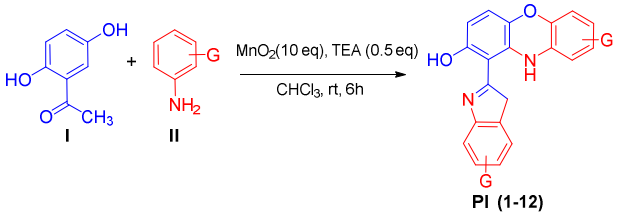
While dioxane showed no improvement in yield (**Table 1**, **entry 3**), dichloroethane showed improvement in yields to 45% (**Table 1**, **entry 4**). Higher temperatures led to drastic decline in the yields (**Table 1**, **entry 2**). Among all, manganese dioxide and 1 equivalent of triethylamine in chloroform as solvent offered the best yields of **1** in **62 %** (**Table 1**, **entry 5**). The yields remained the same when the amount of triethylamine was reduced to 0.5 equiv. The yields of **1** were dropped drastically when the amount of triethylamine was decreased any further.

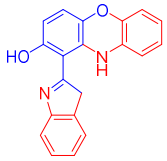
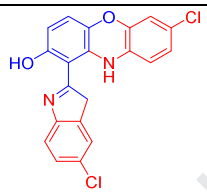
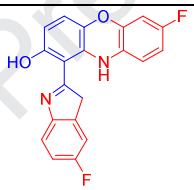
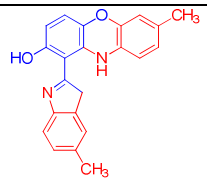
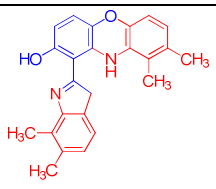
The versatility/limitations of this method were evaluated by examining substrate scope by reacting a varied set of substituted anilines with quinacetophenone (**Table 2**, isolated yields listed) using the conditions outlined above. The results show that R-group substitution can have a significant effect on product yield. For instance, electron-donating methoxy and methyl substituents at 2, 3, 4 positions with respect to the aniline (**Table 2, entries 4-12**) are well tolerated by this reaction. The enhancement of nucleophilicity of carbonyl by electron

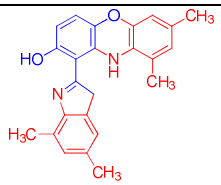
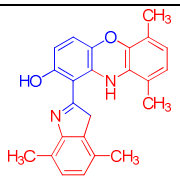
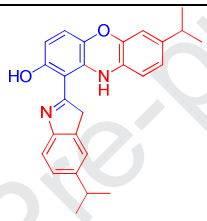
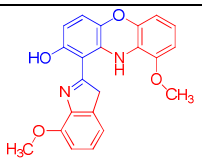
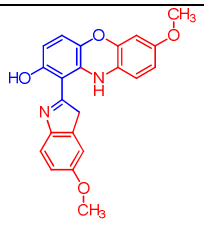
donating character of the methoxy/ methyl group facilitating the imine formation could be a plausible explanation for such OMe and methyl group tolerance in *o*, *m* and *p*-positions. It was reported earlier that trimethoxyphenyl moiety is one of the important structural units present in several known natural antimitotic agents like combretastatin A-4, colchicine and podophyllotoxin. The trimethoxyphenyl moiety apparently binds at the colchicine site of tubulin.[16]We believed that incorporation of such a crucial structural feature of lead anticancer compounds into our 1-(2′-indolyl)-phenoxazines may result in a potent anticancer compound. Thus **12** (**entry 12**) was synthesized with trimethoxy susbstituents which was obtained in similar yields.

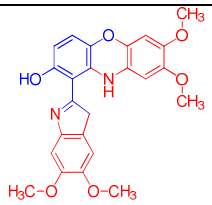
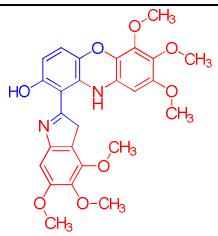
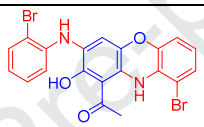
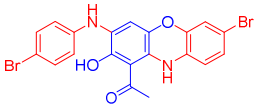
It is also interesting to note that when G is a halogen (Cl or F), (**entries 2-3**) the yield drops substantially (40%; the inductively withdrawing character of Cl and F may have had the opposite effect of the -OMe group). However, presence of Br has not yielded any product (**entry 13, 14**). Not surprisingly, when G is an electron withdrawing group, the reaction did not afford any product. Thus, anilines with substituents like nitro, cyano and ester afforded no products (**entries 15-22**). If nucleophilicity of nitrogen is the criterion, then aryl amines like adenine and 2-amino pyridine (due to electronegativity of nitrogen) must also either not react or afford product in low yields. Thus, we have explored the reaction of quinacetophenone with adenine and 2-amino pyridine to validate our assumption. As expected, no product formation was seen from **1a (**not shown in **Table 1)**.

Table 2. One step synthesis of 1-(indol-2-yl)-phenoxazine hybrids from quinacetophenone and substituted anilines



Entry	-G	Product	Yield
1	H	 **1**	**45**
2	4-Cl	 **2**	**40**
3	4-F	 **3**	**40**
4	4-CH_3	 **4**	**52**
5	2,3-di-CH_3	 **5**	**35**

6	2,4-di-CH_3	 **6**	**52**
7	2,5-di-CH_3	 **7**	**40**
8	4-^{i}Pr	 **8**	**42**
9	2-OMe	 **9**	**50**
10	4-OMe	 **10**	**45**

11	3,4-di-OMe	 **11**	**60**
12	3,4,5-tri-OMe	 **12**	**62**
13	2-Br	 **13**	**48**
14	4-Br	 **14**	**52**
15	4-CH_3;3-NO_2	NR	-
16	2-CH_3;4-NO_2	NR	-
17	2-NO_2	NR	-
18	3-NO_2	NR	-
19	4-NO_2	NR	-
20	3-CF_3	NR	-
21	4-COOMe	NR	-
22	2-CN	NR	-

NR: no reaction

Plausible mechanism

To study the mechanism of the reaction we have made ESI-MS spectrometry as techniques of our choice, in order to establish the most plausible mechanism. NMR spectroscopy alone was useful only in validating the products formed.**Figure 2 (A-E)** shows the mass spectra of the reaction at different intervals. We have captured reaction intermediates by spectroscopic techniques and established the most probable reaction pathway.

In presence of manganese dioxide, quinacetophenone is oxidized to give 2-acetyl-[1,4]-benzoquinone **A,**[3a, 3b]which is most prominently, attacked by nucleophiles selectively at C-6 providing 6-substituted quinacetophenone, Thus, 1 min following the addition of aniline, the presence of a prominent peak at m/z 244. 0977 amu corresponding to **[B-I +H]**$^{+}$ in **Figure 2A** provided key information on the composition of intermediate **B-I**, thus allowing the intermediate to be associated with the formation of the aza-Michel addition product for the eventual formation of the desired product.[3b]

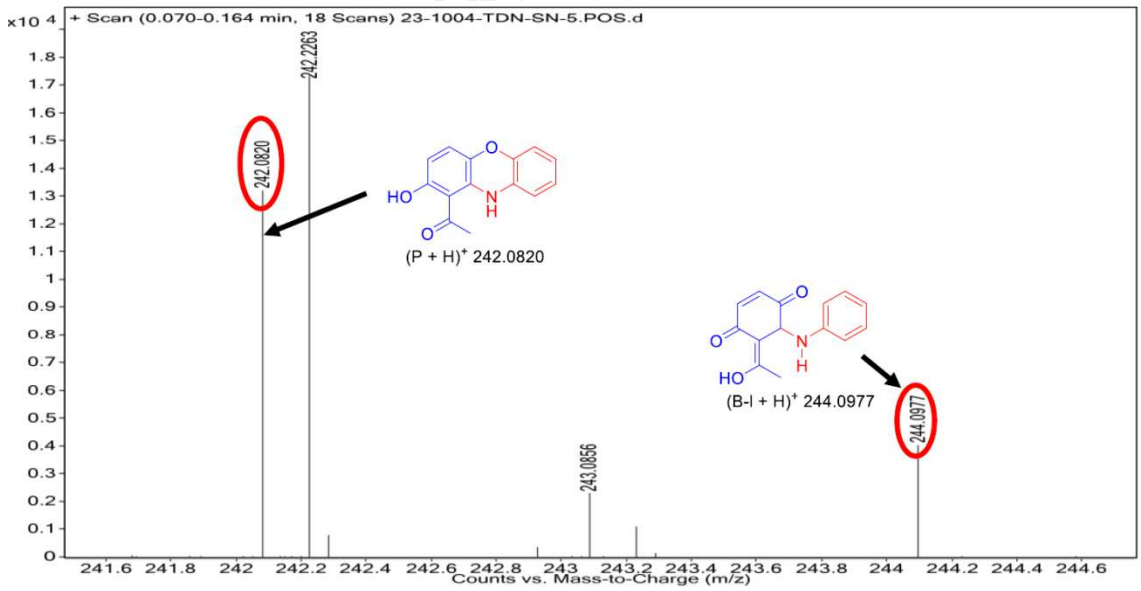


Figure 1A.ESI-MS of the reaction mixture, 1 min after the addition of aniline to quinacetophenone. The signals due to species [B-l +H] $^{+}$ [P + H] $^{+}$ are encircled.

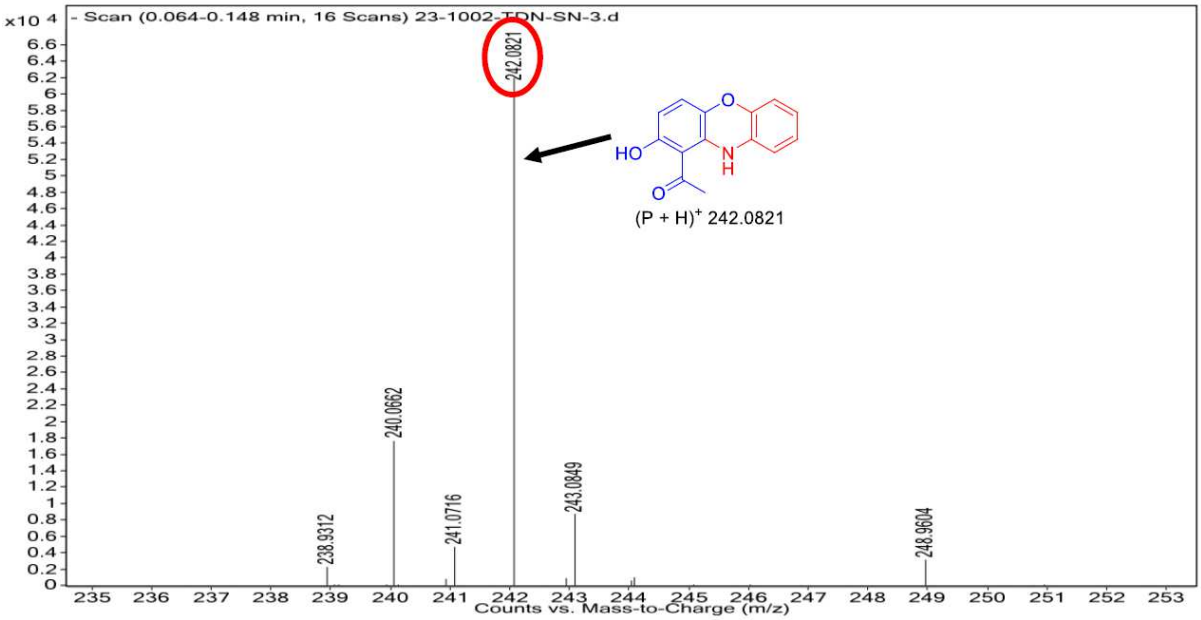


Figure 2B.ESI-MS of the reaction mixture, 1.5 h after the addition of aniline to quinacetophenone. The signals due to species $[P + H]^+$ are encircled.

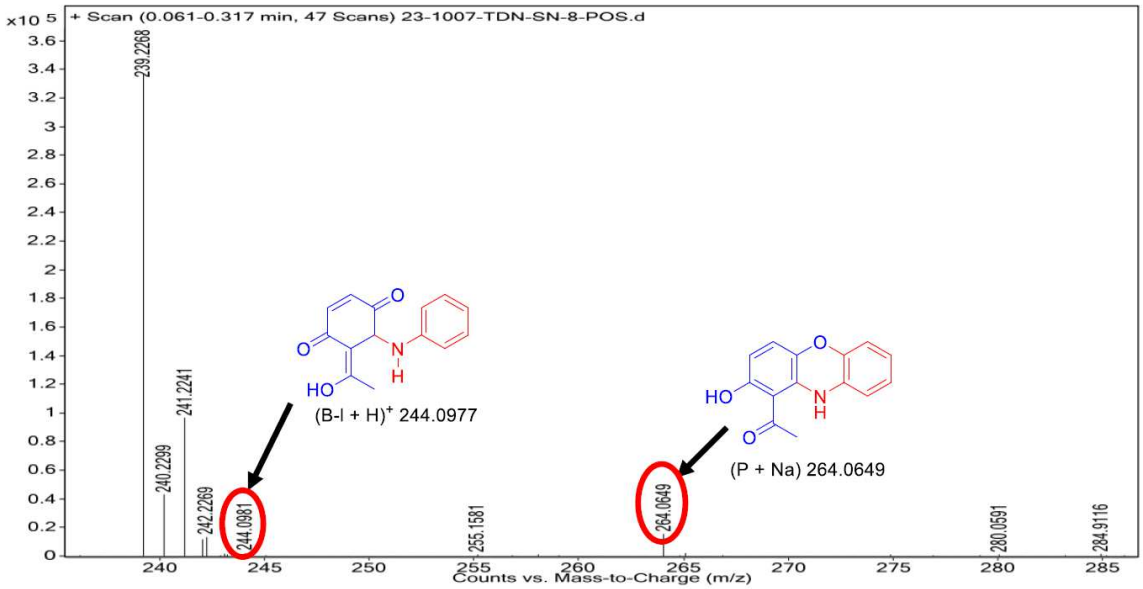


Figure 2C. ESI-MS of the reaction mixture, 5h after the addition of aniline to quinacetophenone. The signals due to $[B\text{-}l + H]^+$ and $[P + H]^+$ are encircled**.**

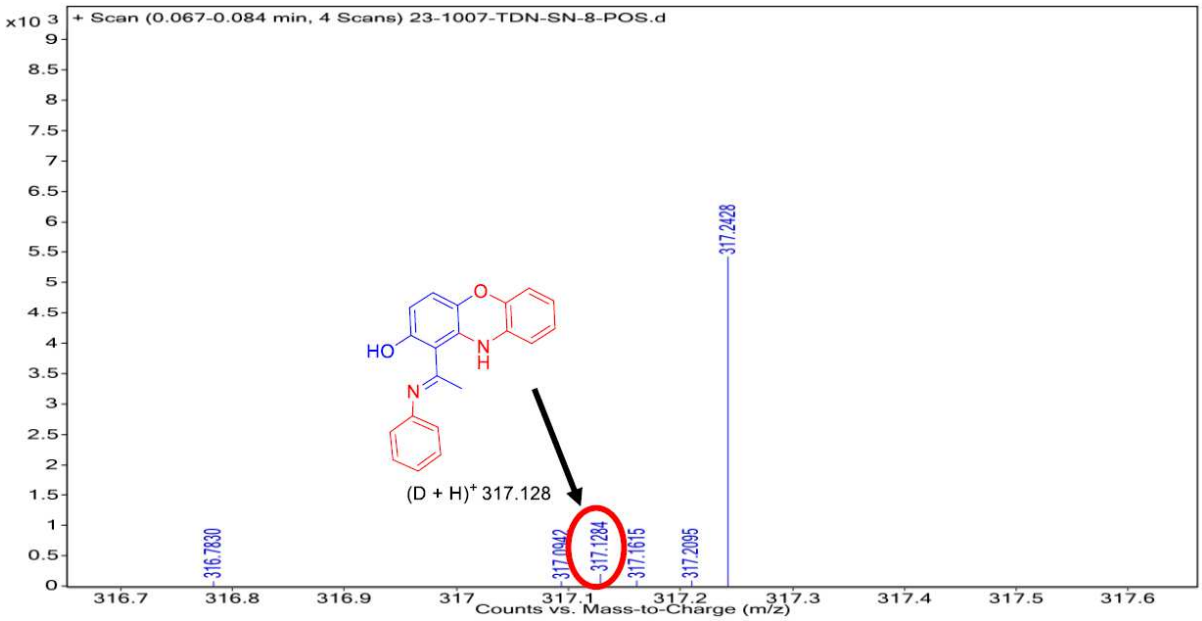


Figure 2D.ESI-MS of the reaction mixture, 5h after the addition of aniline to quinacetophenone. The signals due to $[D+H]^+$ are encircled.

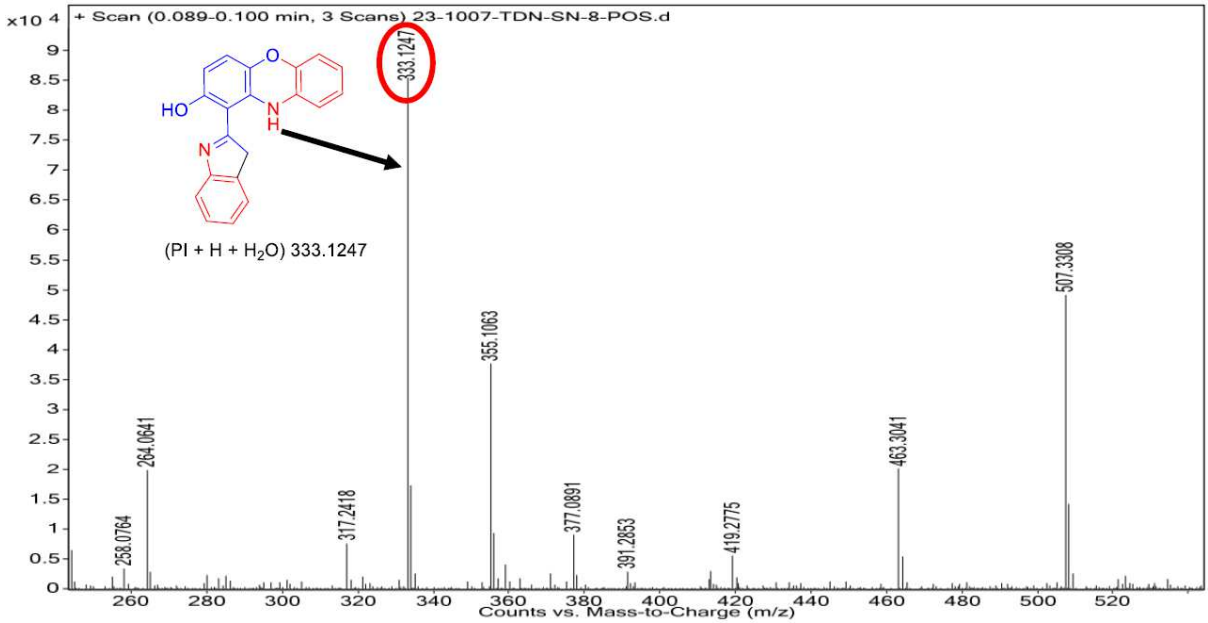
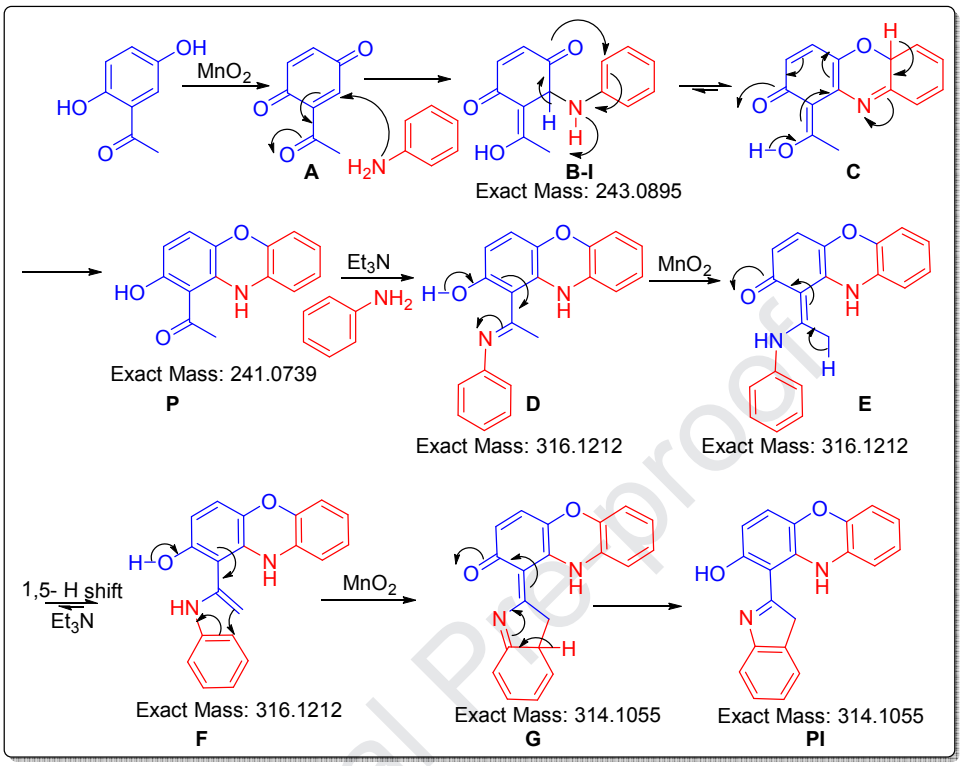


Figure 2E. ESI-MS of the reaction mixture 5h after the addition of aniline to quinacetophenone. The signals due to $[PI + H_2O + H]^+$ encircled.

The peak at m/z 244.0977 amu $[B\text{-}l + H]^+$ was found to coexist with the second molecular ion peak at m/z 242.0820 amu corresponding to $[P + H]^+$ which is indicative of rapid cyclization of aza-Michael adduct to phenoxazine P in accordance with the mechanism proposed in

Scheme 1(Figure 2A) Further, ESI-MS of sample after 1.5 h showing a tall peak at m/z 242.0821 confirmed the formation of P(Phenoxazine) in the reaction mixture **(Figure 2B)** The sample taken after 5 h showed the less intense peaks corresponding to $[B\text{-}l + H]^+$ and $[P + Na]^+$ indicating a decreasing concentration of the intermediates **(Figure 3C).** The ESI-MS of the reaction mixture after 5 h also showed the rising of peak at m/z 317.1284 amu corresponding to $[D + H]^+$ indicating the initiation of imine formation **(Figure 2D).** The strong peak at m/z 333.1247 amu corresponding to $[PI + H_2O + H]^+$ after 5h is indicative of the formation of PI **(Figure 2E).** There are peaks detected by ESI-MS, which may be species that may have formed during the ionization process and may be slightly different from the true species present in the reaction.

The most plausible mechanism delineated for the reaction to account for the formation of product is shown in **Scheme 2**. The initial event may be considered as the nucleophilic attack of amine in an aza-Michael fashion on 1-acetyl benzoquinone A, to generate an intermediate B-I. Intermediate B-I undergoes subsequent electro cyclization to form C and undergoes eventual rearomatization to form the initial phenoxazine P. P in the presence of triethylamine reacts with a second molecule of aniline to form a phenolic Schiff base D. D on oxidation in the presence of MnO_2 forms a transient enamine E, which furtherundergoes 1,5-H shift to form a cyclization precursor F. F on annulation forms an exocyclic imine G which finally rearomatizes to the final product PI(Phenoxzine-Indole).



Scheme 1. Plausible mechanism

Biological activity

The newly synthesized indolyl phenoxazine derivatives **1-12** were screened for their cytotoxicity against various human cancer cell lines A549 (human lung cancer), MG-63 (human bone cancer), BT-474 (human brest cancer), HepG2 (human hepatic cancer), HCT-116 (human colon cancer), along with normal lung epithelial tissue cells (L-132) purchased from American Type Culture Collection (ATCC) USA, by utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[16] The IC_{50} (μM) values from the *in vitro* cytotoxic evaluation of the synthesized derivatives **1-12**as well as standard drugs are reflected in Table 3.Remarkably, the tested derivatives showed good to moderate cytotoxicity on the selected cancer cell lines. Preliminary results indicated that among all the derivatives **6,7**and**8** showed significant antiproliferative activities against the tested cancer

cell linespointing towards hydrophobicity which describes the tendency towards participation in the London dispersion forceswhich are quite important for hydrophobicsubstances tointeract within themselves and other neighbouring moieties. Compound **8** possessing 4-iso-propyl substituent was most active with an IC_{50} value of **3.71 ± 0.57 μM**, followed by compound **6**(**4.43 ± 0.64 μM**)with 3,4-dimethyl substituent and compound **7**(**6.08 ± 0.78 μM**)with 2,5-dimethyl substituent against A-549. Compound **7**was also found to be fairly active against HCT-116 (**9.19 ± 0.19 μM**).compound **2, 3** and **9** with the F, Cl and 2-methoxy respectively displayed weak antiproliferative activities with the IC_{50} values > 30.0 μM. Since halogen substituents increase the lipohilicity, the molecule might probably assume a bigger size.Compound**1, 4, 5** which areeither unsubstitutedwith hydrophobic substituents on 2-, 3-, and 4- positions**,** and**10, 11**with methoxy substitutionswere found to be inactive.Thus, steric bulk at4-, and 5- positions of phenoxazine seem crucial for activity. (not shown)

Table 3. *In vitro* anticancer activity of compounds **1-12**.

Compounds	A549[b]	MG-63[c]	BT-474[d]	HepG2[e]	HCT-116[f]	L132[g]
2	>30	>30	>30	>30	>30	>30
3	>30	>30	>30	>30	>30	>30
8	**3.71 ± 0.57**	19.19 ± 0.17	15.94 ± 1.42	>30	12.04 ± 1.78	24.11 ± 0.94
12	11.08 ± 1.02	14.25. ± 1.14	>30	19.52 ± 1.09	20.17 ± 0.08	10.23 ± 0.32
9	>30	25.55 ± 1.02	28.56 ± 1.04	25.33 ± 0.52	18.19 ± 1.01	15.36 ± 1.20
7	**6.08 ± 0.78**	21.04 ± 0.95	**7.13 ± 0.78**	16.15 ± 1.06	**9.19 ± 0.19**	10.29 ± 0.12
6	**4.43 ± 0.64**	19.15 ± 0.97	25.48 ± 0.94	13.55 ± 0.18	18.55 ± 0.44	13.96 ± 0.51
Doxorubicin[i]	1.08 ± 0.28	0.78 ± 1.55	2.81 ± 1.17	2.44 ± 0.17	0.68 ± 0.11	0.95 ± 0.84

[a] 50% Inhibitory concentration after 72 h of drug treatment, [b] human lung cancer, [c] human bone cancer, [d] human brest cancer, [e] human hepatic cancer, [f] human colon cancer, [g]human lung epithelial

cells, [i] Reference compound. All the values are expressed as Mean ± SEM in which each treatment was performed in triplicate wells.

DNA-Nanodrop method

DNA intercalation is determined by nanodrop spectrophotometric analysis which measures the absorbance and calculates the concentration of nucleic acids (260 nm). The blue and red shifts, hypo and hyperchromic effects, are spectral properties of DNA-drug interaction, which are closely related with the double helix structure [17a]. The possibility of interaction of ligands to DNA can be investigated according to changes in the absorption spectra before and after the reaction. In general, an intercalation induces the hyperchromicity at 260 nm. [17b] Such a rise in the absorbance on intercalation is observed owing to the possible enhancement of axial length of the DNA. Hypochromicity at the maximum absorption of DNA indicates the compaction of DNA due to the electrostatic interaction whereas binding of certain groove binders cause little or no change in the absorbance.[18b, 18c].

In our present experiment, the CT-DNA concentration was fixed at 50×10^{-6} M, in TAE buffer and incubated it with test compound (5µM), Ethidium Bromide (1 µM) for ten minutes. TAE buffer was used as blank and absorbance was determined by using Nano Drop™ 2000/2000c Spectrophotometer (Thermo fisher scientific, USA). We have observed that in comparison to the control DNA, Ethidium Bromide, a known intercalator and compound **8** have shown an increase in the intensity of absorbance at 260nm, indicating their binding ability to bind to DNA thereby validating the possibility of DNA intercalation.

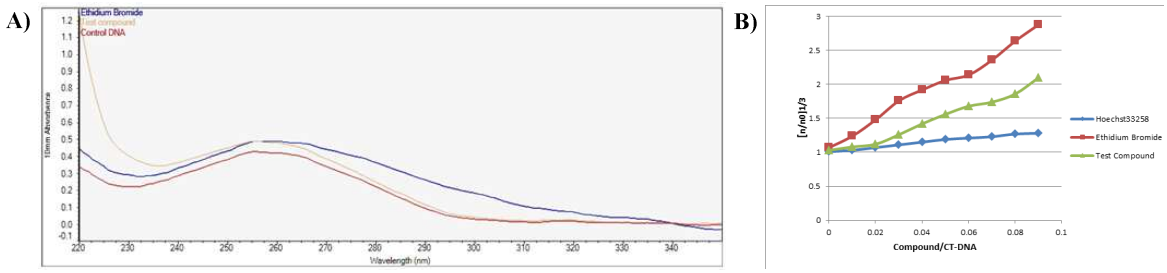


Figure 2. DNA intercalation studies A) Spectrophotometric analysis by nanodrop spectrophotometer for determination of absorbance: EtBr and Compound **8** have enhanced DNA absorptivity compared to control. B) Relative viscosity measurements to determine the DNA intercalation: Hoechst-33258 and EtBr used as reference standards.

DNA intercalation using viscosity measurements

We have performed viscosity measurements to track the changes of DNA viscosity in the presence of compound **8** in order to validate the internal binding to DNA. When a ligand binds externally either in the major grove or minor grove, a slight bending of DNA helix leads to decrease in relative length of DNA as observed by a decrease in its viscosity. However, when the ligand intercalates between the DNA bases, it results in local unwinding and lengthening of DNA leading to an increase in viscosity. Here, we have used EtBr, an intercalator as a positive control along with Hoechst-33258, a known DNA minor groove binder and compound **8** in our viscosity measurements to compare the changes in viscosity on their reaction with DNA. In the viscosity experiments we have conducted, although the viscosity of compound **8** was not as high as that of Ethidium bromide (EtBr), the DNA showed higher viscosity as compared to Hoechst 33258. Viscosity measurements were conducted using a Lovis 2000 M/ME Rolling-ball viscometer (Anton Paar GmbH, Graz, Austria), based on the falling ball principle. CT-DNA concentration was fixed at 50×10^{-6} M, in 100 mMTris-HCl (pH 7.4). Compound **8**was added at various concentrations to CT-DNA solution. A calibrated 1.59 mm glass capillary containing a steel ball was filled with the sample and viscosity was measured by measuring the ball falling time at angles in the range from 20^{o} to 70^{o}. Ethidium bromide, Hoechst-33258 were used as controls. Data was

represented graphically as $(\eta/\eta_0)^{1/3}$ vs. the ratio of the concentration of the hybrid to CT-DNA, where ŋ is the viscosity of CT-DNA in the presence of the derivative and η_0 is the viscosity of CT-DNA solution.

Conclusion

In conclusion, we have demonstrated the utility of the versatile, easily accessible building block quinacetophenone, for the synthesis of 1-(indol-2-yl)-phenoxazines via C-N formation as a key step. ESI-MS was used as a tool to identify the intermediates in order to establish the mechanism. The method is tolerant of electron donating groups.*In vitro* cytotoxic evaluation for these derivatives were screened against the seleted panel of human cancer cell lines, from preliminary screeing data it was clear thatcompound **8** displyaed signifiacnt $\mathbf{IC_{50}}$ value of **3.71 ± 0.57 *μ*M** on **A-549** cell line. DNA nanodrop method and viscosity experiments have ben conducted to study the changes in absorbances and viscosity of DNA to confirm the DNA intercalating ability of compound **8**.

Experimental section

General Methods: All reactions were carried out under an inert atmosphere with dry solvents unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) on silica gel plates (60 F254), using UV light detection. Visualization of the spots on TLC plates was achieved either by UV light or by staining the plates in 2, 4-Dinitro phenyl hydrazine/ Ninhydrin stains and charring on a hot plate. Flash chromatography was performed on silica gel (200-400 mesh) using distilled hexane, ethyl acetate, dichloromethane. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solution by using Bruker 500 MHz NMR spectrometer. Chemical shifts are reported as δ values relative to internal $CDCl_3$ δ 7.26 or TMS δ 0.0, DMSO-d_6 δ 2.5 for ^{1}H NMR and $CDCl_3$ δ 77.0, DMSO-d_6 δ 39.52 for ^{13}C NMR. ^{1}H NMR data were recorded as follows: chemical shift [multiplicity, coupling constant(s) *J* (Hz), relative integral] where multiplicity is defined as s (singlet), d (doublet), t (triplet), q

(quartet), dd (doublet of doublet), m (multiplet), bs (broad singlet). FT-IR spectra were recorded on PerkinElmer spectrometer. High-resolution mass spectra (HR-MS) $[ESI]^+$ were obtained using Agilent Q-TOF mass spectrometer 6540 series instrument

General procedure for the synthesis of 1-(indol-2-yl)-phenoxazine derivative. (1-14):

A suspension of quinacetophenone I (0.152 g, 1 mmol) and MnO_2 (0.860g, 10 mmol) is cooled in an ice bath to 0 °C under constant stirring. Anilines II (0.182 mL, 2.0 mmol) in anhydrous chloroform and triethylamine (0.068 mL, 0.5 mmol) were added and stirred at rt for 6h. The mixture was filtered through celite. Removal of the solvent under reduced pressure followed by column chromatography (5% EtOAc/Hexane) yielded pure 1-(indol-2-yl)-phenoxazines (PI) in moderate to good yields.

*1-(3H-indol-2-yl)-10H-phenoxazin-2-ol (**1**)*

Orange solid, Yield (45%), m. p. = 165-167 °C, R_f 0.5 (30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, $CDCl_3$): δ 8.19 (s, 1H), 7.43–7.35 (m, 4H), 7.30–7.21 (m, 4H), 7.13 (d, *J* = 7.5 Hz, 2H), 6.04 (s, 1H), 2.65 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.58, 177.88, 146.43, 139.22, 137.06, 129.71, 129.05, 127.05, 126.17, 124.28, 122.79, 107.80, 99.14, 32.61. IR (neat, cm^{-1}): 3291, 3046, 2804, 1649, 1626, 1615, 1509, 1233, 1156. HRMS *m/z* calculated for $C_{20}H_{14}N_2O_3$ $[M + H_2O + H]^+$: 333.1239 found: 333.1238.

*7-chloro-1-(5-chloro-3H-indol-2-yl)-10H-phenoxazin-2-ol (**2**)*

Orange solid, Yield (40%), m. p. = 203-205 °C, R_f 0.4 (30:70 Ethyl acetate: Hexane); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.12 (s, 1H), 7.38 (d, *J* = 8.7 Hz, 2H), 7.32 (d, *J* = 8.65 Hz, 2H), 7.17 (d, *J* = 8.7 Hz, 2H), 7.04 (d, *J* = 8.61 Hz, 2H), 5.89 (s, 1H), 2.59 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.42, 177.72, 162.35, 161.66, 160.38, 159.69, 146.89, 134.74, 132.84, 126.04, 125.97, 125.07, 125.01, 119.08, 166.62, 166.09, 115.90, 107.38, 32.63. IR (neat, cm^{-1}): 3289, 3076, 2954, 2849, 2917, 1652, 1615, 1593, 1229, 1170, 753. HRMS *m/z* calculated for $C_{20}H_{12}Cl_2N_2O_3$ $[M + H_2O + Na]^+$:423.0279 found: 423.0269.

*7-fluoro-1-(5-fluoro-3H-indol-2-yl)-10H-phenoxazin-2-ol (**3**)*

Orange coloured solid, Yield (40%), m. p = 200-202 ^{o}C, R_f 0.5 (30:70 Ethyl acetate: Hexane); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.08 (s, 1H), 7.26–7.03 (m, 8H), 5.86 (s, 1H), 2.64 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.42, 177.72, 162.35, 161.36 (d, *J* = 247.23 Hz), 160.67 (d, *J* = 247.67 Hz), 158.48, 146.88, 135.12, 132.87, 129.27, 126.01 (d, *J* = 8.37 Hz), 125.05 (d, *J* = 8.26 Hz), 122.05, 119.63, 116.80 (d, *J* = 23.16 Hz), 116.01 (d, *J* = 23.16 Hz), 107.76, 98.96, 32.66. IR (neat, cm^{-1}): 3304, 3081, 2923, 2852, 1651, 1615, 1289, 1185, 734. HRMS *m/z* calculated for $C_{20}H_{12}F_2N_2O_3$ $[M + H_2O + H]^+$:369.1051 found: 369.1051.

*7-methyl-1-(5-methyl-3H-indol-2-yl)-10H-phenoxazin-2-ol (**4**)*

Orange solid, Yield (52%), m. p. = 198-203 ^{o}C, R_f 0.5, (30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, $CDCl_3$): δ 8.16 (s, 1H), 7.21 (d, *J* = 10 Hz, 2H), 7.16–7.11 (m, 4H), 7.01 (d, *J* = 10 Hz, 2H), 5.98 (s, 1H), 2.65 (s, 2H), 2.35 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.45, 177.83, 146.70, 136.95, 136.20, 134.40, 130.24, 129.67, 123.97, 122.76, 32.62, 21.11, 20.99. IR (neat, cm^{-1}): 3316, 2922, 2852, 1743, 1645, 1621, 1287, 1191. HRMS *m/z* calculated for $C_{22}H_{18}N_2O_3$ $[M + H_2O + H]^+$: 361.1552 found: 361.1552.

*1-(6,7-dimethyl-3H-indol-2-yl)-8,9-dimethyl-10H- phenoxazin-2-ol (**5**)*

Orange solid, Yield (35%), m. p. = 169-173 ^{o}C, R_f0.5 (30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, $CDCl_3$): δ 8.00 (s, 1H), 7.15–7.04 (m, 5H), 6.85 (d, *J* = 7.5 Hz, 1H), 5.50 (s, 1H), 2.72 (s, 2H), 2.31 (s, 6H), 2.16 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.14, 177.84, 148.18, 138.69, 137.94, 138.02, 135.01, 130.94, 132.08, 128.94, 128.86, 126.35, 125.82, 122.80, 122.44, 107.37, 99.14, 32.60, 20.41, 14.41, 13.98. IR (neat, cm^{-1}): 3314, 2919, 1737, 1651, 1614, 1515, 1386, 1217. HRMS *m/z* calculated for $C_{24}H_{22}N_2O_3$ $[M + H_2O + H]^+$:389.1865 found: 389.1862.

*1-(5,7-dimethyl-3H-indol-2-yl)-7,9-dimethyl-10H- phenoxazin -2-ol (**6**)*

Orange solid, Yield (52%), m. p. = 202-207^{o}C, R_f 0.5, (30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.03 (d, *J* = 8.5 Hz, 2H), 6.97 (d, *J* = 8.0 Hz, 2H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.80 (d, *J* = 7.5 Hz, 1H), 5.51 (s, 1H), 2.64 (s, 2H), 2.24 (d, *J* = 4.5 Hz, 6H), 2.15 (d, *J* = 8.0 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.15, 177.81, 147.85, 137.10, 132.85, 132.50, 132.03, 131.46, 127.63, 127.15, 124.45, 99.99, 32.62, 21.03, 20.95, 17.93, 17.61. IR (neat, cm^{-1}): 3313, 2922, 2652, 1731, 1560, 1515. HRMS *m/z* calculated for $C_{24}H_{22}N_2O_3$ $[M + H_2O + H]^+$: 389.1865 found: 389.1865.

*1-(4,7-dimethyl-3H-indol-2-yl)-6,9-dimethyl-10H-phenoxazin -2-ol (**7**)*

Orange solid, Yield (40%), m. p. = 188-193^{o}C, R_f 0.3 (30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, $CDCl_3$): δ 8.00 (s, 1H), 7.16 (d, *J* = 10.0, 7.5 Hz, 1H), 7.12 (d, *J* = 15 Hz, 1H), 7.05 (s, 1H), 7.00 (d, *J* = 7.5 Hz, 2H), 6.81 (s, 1H), 5.66 (s, 1H), 2.70 (s, 2H), 2.32 (d, *J* = 15 Hz, 6H), 2.23 (d, 13.5 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.27, 177.83, 147.56, 138.39, 137.00, 136.96, 136.28, 135.03, 131.21, 130.57, 129.75, 129.26, 128.14, 127.91, 125.10, 124.86, 107.32, 99.96, 32.56, 20.93, 20.85, 17.57, 17.25. IR (neat, cm-1): 3254, 3218, 2922, 2853, 1695, 1615, 1471, 1238, 1121. HRMS *m/z* calculated for $C_{24}H_{22}N_2O_3$ $[M + H_2O + H]^+$: 389.1865 found: 389.1867.

*7-isopropyl-1-(5-isopropyl-3H-indol-2-yl)-10H-phenoxazin-2-ol (**8**)*

Orange solid Yield (42%), m. p. = 148-151°C, R_f 0.4, (30:70 Ethyl acetate: Hexane); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.11 (s, 1H), 7.18 (d, *J* = 4.0 Hz, 2H), 7.17 (d, *J* = 8.5 Hz, 2H), 7.09 (d, *J* = 8.5 Hz, 2H), 6.98 (d, *J* = 8.5 Hz, 2H), 5.94 (s, 1H), 2.87-2.81 (m, 2H), 2.58 (s, 2H), 1.18 (dd, *J* = 2.0, 2.0 Hz, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.49, 177.82, 147.81, 147.17, 146.68, 136.82, 134.66, 127.62, 127.03, 124.06, 122.79, 107.70, 99.15, 33.72, 32.60, 23.87. IR (neat, cm^{-1}): 3218, 2960, 2868, 1739, 1693, 1610, 1635, 1576, 1555, 1417, 1261, 1149. HRMS *m/z* calculated for $C_{26}H_{26}N_2O_3$ $[M + H_2O + H]^+$: 417.2178 found: 417.2175.

*9-methoxy-1-(7-methoxy-3H-indol-2-yl)-10H-phenoxazin-2-ol (**9**)*

Orange solid, Yield (50%), m. p. = 150-155^{o}C, R_f 0.5,(30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, $CDCl_3$): δ 8.53 (s, 1H), 7.30 (d, *J* = 8.75 Hz 1H), *7*.27 (m, 2H), 7.16 (d, *J* = 8.35 Hz 1H), 7.10-7.02 (m, 2H), 6.93-6.83 (m, 4H), 6.06 (s, 1H), 3.85 (s, 3H), 3.74 (s, 3H), 2.59 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ178.31, 177.72, 158.49, 158.02, 156.36, 131.96, 129.66, 125.33, 125.04, 124.68, 119.34, 119.05, 115.52, 114.94, 114.29, 113.64, 107.42, 98.89, 55.52, 55.44, 32.64. IR (neat, cm^{-1}): 3318, 2924, 2853, 1689, 1639, 1590, 1462, 1211. HRMS *m/z* calculated for $C_{22}H_{18}N_2O_5$ [M + H_2O + H] $^{+}$: 393.1450 found: 393.1452.

*7-methoxy-1-(5-methoxy-3H-indol-2-yl)-10H-phenoxazin-2-ol (**10**)*

Orange solid, Yield (45%), m.p 172-176°C, R_f 0.4(30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, DMSO-d_6): δ 8.05 (s, 1H), 7.09 (d, *J* = 8.5 Hz, 2H), 6.98 (d, *J* = 8.0 Hz, 2H), 6.86 (d, *J* = 9.0 Hz, 2H), 6.80 (d, *J* = 8.5Hz, 2H), 5.81 (s, 1H), 3.74 (d, *J* = 6.5 Hz, 6H), 2.62 (s, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 178.28, 178.11, 152.68, 147.00, 128.37, 128.07, 127.62, 126.29, 125.72, 124.20, 121.32, 120.78, 112.64, 112.21, 97.78, 56.32, 56.12, 32.51. IR (neat, cm^{-1}): 3217, 2923, 2840, 1688. HRMS *m/z* calculated for $C_{22}H_{18}N_2O_5$ [M + H_2O + H] $^{+:}$ 393.1450 found: 393.1448.

*1-(5,6-dimethoxy-3H-indol-2-yl)-7,8-dimethoxy-10H-phenoxazin -2-ol (**11**)*

Orange coloured solid, Yield (60%), m. p. = 192-195^{o}C, R_f 0.5 (30:70 Ethyl acetate: Hexane);^{1}H NMR (500 MHz, DMSO-d_6): δ 9.38 (s, 1H), 7.01 (d, *J* = 9.0 Hz, 2H), 6.90 (d, *J* = 8.5 Hz, 2H), 6.81 (s, 1H), 6.70 (d, *J* = 7.5 Hz, 1H), 5.73 (s, 1H), 3.77 – 3.71 (m, 12H), 2.26 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.63, 178.47, 149.60, 149.06, 148.49, 147.73, 147.48, 132.60, 130.95, 116.87, 112.55, 111.91, 109.41, 109.23, 109.16, 96.27, 79.64, 56.23, 56.15, 56.10, 56.02, 32.43. IR (neat, cm^{-1}): 3292, 2927, 2837, 1647, 1605, 1594, 1557, 1513, 1295, 1262, 1162. HRMS *m/z* calculated for $C_{24}H_{22}N_2O_7$ [M + H_2O + H] $^{+}$: 453.1662 found: 453.1665.

6,7,8-trimethoxy-1-(4,5,6-trimethoxy-3H-indol-2-yl)-10H-phenoxazin-2-ol (12)

Orange solid, Yield (62%), m. p. = 125-130 ^{o}C, R_f 0.5 (30:70 Ethyl acetate: Hexane); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.12 (s, 1H), 6.46 (s, 2H), 6.35 (s, 2H), 6.02 (s, 1H), 3.85-3.82 (m, 18H), 3.82 (s, 6H), 2.64 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ178.94, 178.57, 153.72, 153.68, 153.27, 148.04, 136.32, 136.10, 135.92, 135.41, 133.79, 124.33, 123.10, 119.76, 109.79, 109.37, 108.72, 102.76, 102.44, 102.35, 96.96, 6062, 60.57, 56.57, 56.44, 32.36. IR (neat, cm^{-1}): 3258, 2924, 2852, 1586, 1452, 1416, 1228, 1182, 1123, 1042. HRMS *m/z* calculated for $C_{26}H_{26}N_2O_9$ $[M + H_2O + H]^{+:}$513.1873 found: 513.1871.

*1-(9-bromo-3-((2-bromophenyl)amino)-2-hydroxy-10H-phenoxazin-1-yl)ethan-1-one (**13**)*

Brown Solid; Yield- 48%, m. p. = 202-202 °C, R_f 0.3 (30:70 Ethyl acetate: Hexane); ^{1}H NMR (500 MHz, DMSO- d_6): δ 12.34 (s, 1H), 9.39 (s, 1H), 7.80 (d, *J* = 8.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 1H), 7.51 (t, *J* = 8.0 Hz, 1H), 7.46 (d, *J* = 6.5 Hz, 1H), 7.37 (t, *J* = 7.6 Hz, 1H), 7.32 (d, *J* = 7.0 Hz, 2H), 7.23 (t, *J* = 7.6 Hz, 1H), 5.19 (s, 1H), 2.44 (s, 3H), ^{13}C NMR (125 MHz, DMSO- d_6): δ 200.27, 178.31, 178.02, 148.45, 138.84, 136.37, 133.95, 133.08, 129.49, 129.05, 128.65, 128.39, 127.95, 120.71, 119.56, 108.86, 98.03, 32.59. IR (neat, cm^{-1}): 3277.84, 3003.17,2 779.41, 1737.20, 1356.93, 1288.47, 1027.99, 759.24, 661.20. HRMS *m/z* calculated for $C_{20}H_{14}Br_2N_2O_3$ $[M + H]^{+}$: 490.9429 found 490.9431.

*1-(7-bromo-3-((4-bromophenyl)amino)-2-hydroxy-10H-phenoxazin-1-yl)ethan-1-one (**14**)*

Red Solid; Yield- 52%, m. p. = 215-217 °C, R_f 0.3 (30:70 Ethyl acetate: Hexane); ^{1}H NMR (500 MHz, DMSO- d_6): δ 11.07 (s, 1H), 9.60 (s, 1H), 7.61 (s, 1H), 7.47 - 7.38 (m, 5H), 7.31 (t, *J* = 8.0 Hz, 1H), 7.19 (d, *J* = 8.8 Hz, 1H), 5.86 (s, 1H), 2.32 (s, 3H), ^{13}C NMR (125 MHz, DMSO- d_6): δ 199.81, 179.56, 179.04, 147.46, 139.92, 131.69, 131.08, 129.31, 128.87, 127.12, 126.8, 123.67, 123.0, 122.3, 121.74, 110.26, 97.69, 32.30. IR (neat, cm^{-1}): 3300.03, 3000.28, 2950.35, 1737.20, 1653.44, 1310.23, 1215.30, 1000.25, 820.30, 650.80. HRMS *m/z* calculated for $C_{20}H_{14}Br_2N_2O_3$ $[M + H]^{+}$: 490.9429 found 490.9425.

Pharmacology

Cell culture

Human cancer lines such as A549 (human lung cancer), MG-63 (human bone cancer), BT-474 (human brest cancer), HepG2 (human hepatic cancer), HCT-116 (human colon cancer), along with human lung epithelial tissue cells (L-132) were procured from National Centre for cell science (NCCS) Pune India. All cells were maintained with appropriate DMEM and RPMI 1640 media (Sigma Aldrich) cells were also supplemented with 10% Fetal bovine serum stabilized with 1% antibiotic-anti mycotic solution (Sigma Aldrich) in CO2 incubator at 37 ^{o}C. when cells reached up to 80-90% confluency, they were sub-cultured by using 0.25% trypsin/1mM EDTA for further passage. Compounds were dissolved in DMSO to prepare the stock solution of 10 mM. Further dilutions were made accordingly with respective to media to get required concentration.

Evaluation of *in vitro* cytotoxic assay

Cytotoxicity of synthesized compounds was evaluated by MTTassay. Briefly, cells were seeded in 96-well plates at a density of 4000cells per well in 100 μL of complete medium and allowed to growovernight for attachment onto the wells. Then the cells were treated with various concentrations of the compounds for a period of 72h. After the treatment, 100 µL of MTT (0.5 mg/mL) was added and incubated at 37 °C for 4h. Then MTT reagent was aspirated and the formazan crystals formed were dissolved by the addition of 200 μL of DMSO for 20 mins at 37 °C. The quantity of formazan product wasmeasured by using a spectrophotometric microtiter plate reader(Spectra max, M4 molecular devices, USA) at 570 nm wavelength.Initially, cytotoxicity effects of the synthetic derivatives were screened by MTT assay at 30 μM concentration. Among these, the compoundwhich showed IC_{50} value < 30 μM was used for the dose dependentstudies at various concentrations ranging from 0.78 μM to 30 μM inserial dilutions and the percentage of cytotoxicity was calculated.

DNA Nanodrop method

DNA intercalation is determined by nanodrop spectrophotometric analysis. The intercalatingagents decrease the absorption and increase the wavelengths. In our experiment, weincubated the 50 μM of calf thymus DNA (Sigma-Aldrich, USA) with compound **8,** Ethidium bromide (EtBr) Hoechst 32258 for 10 min at 1 μM concentration. Later, the TAE buffer used as blank and absorbance and concentrations were determined by NanoDrop™ 2000/2000c Spectrophotometer (Thermo fisher scientific, USA).

DNA binding using viscosity measurements

Relative studies were determined by Ostwald viscometer. The titrations were conducted for most active compound **8**, EtBr and Hoechst-33258 at 1.0 μM while and they are added to CT-DNA solution(50 μM) and exposed to viscometer. Here, the DNA solution was prepared in 100 mMTrisHCl (pH 7.0). Data were represented as (ŋ/ŋo)1/3 versus the ratio of the concentration of thecompound **8,** EtBr and Hoechst-33258 to CT-DNA, where ŋ is the viscosity of CTDNA in the presence of intercalating agents and ŋo is the viscosity of CT-DNA alone.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information is available free of charge: experimental procedures, characterization of all new products, copies of ^{1}H NMR, and ^{13}C NMR spectra.

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- An operationally simple reaction between quinacetophenone and aniline derivatives to afforded 1-(indol-2-yl)-phenoxazine hybrids in good yields is presented.
- The most plausible reaction mechanism was established based on the mechanistic studies carried out with Mass spectrometry.
- MTT assay reveals compound **8** to display significant IC50 value of $3.71 \pm 0.57\ \mu M$ on A-549 cell line.
- The ability of **8** to intercalate DNA was also confirmed by DNA Nanodrop method and viscosity experiments.

Declaration of interests

- ✓ ☐The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: