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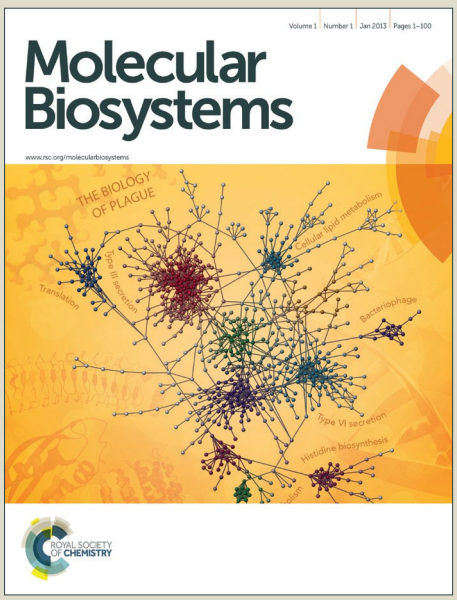





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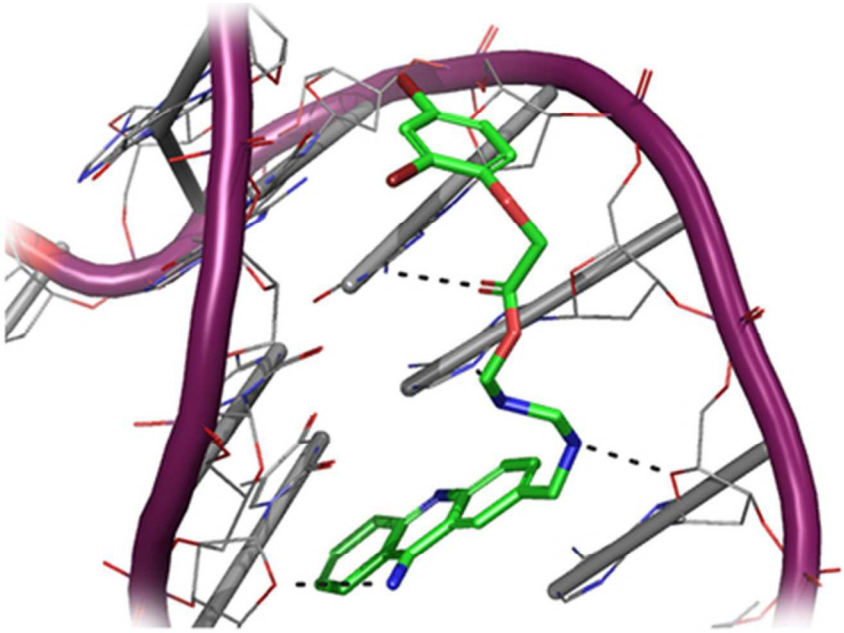

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# Rational design and interaction studies of combilexins towards duplex DNA

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**Abstract**

DNA, the genetic material plays a predominant role in all living organism. Alterations of structure and function this genetic material correlates with complex diseases like cancer. Number of anti cancer drugs exerts their action through binding to DNA. Even though DNA binding compounds exerts genotoxicity, there is a high demand for novel DNA binding molecules as it can be further developed into anti cancer drugs. In the present study, mode of interaction of two compounds like 2,4 D and tacrine has been determined as minor groove binder and intercalator. Later from their binding modes, novel combilexin molecules were designed using computational tools and their mode of binding and affinity towards DNA were determined though a series of molecular modeling experiments like molecular docking, molecular dynamics and binding free energy calculations. The whole studies focuses to the potential effects of combilexins compared to intercalators and minor groove binders. The combilexins deduced from the current studies may be considered as lead compounds for the development of better anti cancer drugs.

**Introduction**

DNA the genetic material have an inevitable role in the central dogma of biology[1]. DNA plays a predominant role in all the living organisms[2]. Alterations in the structure or function of the DNA are always linked with complex diseases. Cancer is one such type of risk which is associated with the alteration or dysfunction of DNA. Obviously the uncontrollable cell division and metastasis leads to the development of various types of cancers. Since it is majorly associated with the malfunction of genetic materials, variety of the anticancer drugs targets DNA and inhibits them from further replications[3, 4]. FDA approved drugs like doxorubicin, actinomycin D, bleomycin, daunorubicin, elsamicin A, epirubicin, mitoxantrone are exert their action by binding to DNA[5]. Hence, potent DNA binding compounds are always significant in the drug discovery

fields as they can be developed into anticancer drugs. Based on the nature of interactions with DNA, the small molecules are broadly divided into groove binders (major and minor) and intercalators[6]. Among groove binders, minor groove binders are most important class of molecules as they can prevent the unwinding and the movement of replication fork and stop the replication of cells[7]. Generally these molecules possess a crescent shaped structure which will be correctly fit into the grooves of the DNA[8]. Similarly the intercalators are expected to intercalate between the base pairs through stacking interactions and stop the DNA replications. Intercalators will generally have an aromatic structure with a specific dimension which enable them to penetrate between the base pairs and stack easily[9].

Apart from these, the combilexins (hybrids of minor groove and intercalator) are another class of DNA binding compounds which exert their action through dual binding modes. Generally in combilexins, groove binding and intercalating functionalities are fused together so as to achieve dual mode of binding[10]. It is considered that combilexins interact more strongly with DNA than any other class of DNA binders[10]. Various reports indicated that combilexins are capable to increase the melting temperature of DNA and expected to have a prolonged residence of time on the DNA than any other individual functionalities like groove binder and intercalator[11]. The DNA topoisomerase inhibitory activity has also been reported for various combilexins[11]. So far, only a small number of combilexins were designed and studied against duplex DNA and majority of them exerts promising anti cancer activities. Over the past few years combilexins were designed and used for various purposes like DNA recognition, anti cancer activities etc[12]. Combilexins that are designed by fusing netropsin/distamycin with acridine and ellipticine are known to pssess potential anti cancer activities[13, 14]. Another combilexin molecule, ‘R-132’ was designed (by combining two intercalating agents with a crescent shaped minor groove binder)

and studied against duplex DNA. The dissociation of 'R-132' from the duplex DNA was already studied and compared with other ligands. These detailed studies also revealed the potential cytotoxic effects and topoisomerase inhibitory activity of 'R-132' [15]. In another study, a set of combilexins were designed by linking oligo pyrrole carboamides with tricyclic DNA intercalators and studied against both duplex DNA as well as topo isomerase[16]. Similarly bis-netropsin-anthraquinone conjugates have been studied against different cancer cell lines[17]. It is reported that one of well studied combilexin 'ThiaNet-GA' was designed by coupling a minor groove binder 'ThiaNet' (thiazole containing analog of netropsin) and an intercalator chromophore GA (glycl-anilinno-9-amino acridine). Many potential activities were identified for this compound[18]. Another combilexin, (a hybrid of an intercalator anthrapyrazoles and a minor groove binder netropsin) exhibited significant inhibitory activity against topoisomerase I and II through DNA binding [19]. For the same reason, designing of novel combilexins with highest DNA binding affinity and with the ability to block different transcription factors are always important in the search for the lead molecules against life threatening diseases like cancer.

In the present study an attempt was made to design a series of combilexins by coupling a minor groove binder and an intercalator. Two molecules such as 2,4-D and tacarine were used for the designing purposes. 2,4-D is a synthetic auxin which enhances the growth of plants at lower concentrations and acting as a herbicide at higher concentrations. Various studies suggested that 2,4-D is capable of crossing intestinal tissues and blood gut barriers[20]. Even though any beneficial activities of this compound on human body has not been well explored, few reports are there which indicates that 2,4-D can potentially block the DNA from further replications. But still their exact mechanism of action is not explained. The selectivity and specificity towards various cancer cells has not been validated for this compound. However a potent anti

inflammatory activity was deduced for this molecule very recently through repositioning studies[21]. Apart from this, a detailed QSAR based studies has also been carried out on 2,4 D to predict its conformational flexibility vs activity and the studies can be effectively utilized for developing novel therapeutic drugs that targets human ubiquitin ligase[22, 23]. Tacrine (9-amino-1,2,3,4-tetrahydroacridine) is a monoamine acridine and it is one of the most effective acetylcholinesterase inhibitor used for the treatment of Alzheimer's disease[24]. Apart from these, reports are there which indicates that tacrine can inhibit topoisomerase and DNA synthesis and induce apoptosis[25]. Some other interesting activities such as inhibition of human liver carboxylesterase and histamine methyl transferase were also reported for tacrine[26]. Since it contains acridine moiety it is expected to block the DNA by intercalating between the bases.

Computational studies are found to be indispensible part of the drug design and discovery field. Over the years various computational resources were used to predict the interaction of molecules in a time saving and cost effective manner. These computational facilities were also used for the identification of various DNA binding molecules [27, 28, 29]. Various computational resources have been exploited in the current study to predict the interaction of two molecules such as 2,4 D and tacrine against duplex DNA. Later a series of novel combilexins were designed through computational studies and effectively screened in the current study.

## Materials and methods

### Isothermal titration calorimetry

In order to check the interaction of 2,4 D and tacarine against duplex DNA, an isothermal titration calorimetry study has been carried out. Two synthetically constructed, HPLC purified double stranded oligo nucleotides 5′- D(CGCGAATTCGCG)-3′ (DNA-1) and 5′-D(CGATCG)-3′ (DNA-2) were purchased from Integrated DNA Technologies, Inc and used without further

purification. Similarly the ligands such as 2,4 D and tacarine were purchased from Sigma Aldrich bengaluru India. The ligands as well as DNA were prepared in phosphate buffer 10 mM containing 200 mM NaCl. Approximately 0.01 mM of DNA and 0.2 mM of ligands were prepared in the same buffer. Prior to the experiment the samples were degassed in order to remove the trapped air. Whole ITC experiments were performed at the temperature 298.15 K using VP-ITC isothermal titration calorimeter from Microcal (Northampton, MA, USA). As a part of the experiment, ligand solutions were added to the cell containing DNA solution using a rotating syringe (at a speed of 307 rpm). Total 29 injections were made in which the volumes of all injections were maintained as 10 µl except the first injection. The volume of the first injection was kept as 2 µl in order to avoid inaccuracy. The time taken for the injection was 10 sec and the time interval between two consecutive injections was set as 180 seconds. Also the reference power set for the experiment was 10 µcal. Finally after the experiment the data was fitted using ORIGIN 7.0 and the following values such as binding constant (K), enthalpy change (ΔH), entropy change (ΔS), and binding free energy (ΔG) were calculated.

**Molecular modeling studies**

In order to predict the atomic level of interaction of 2,4 D and tacrine with DNAs, molecular docking studies were carried out. The three DNA molecules having PDB IDs 443D[30] (Sequence : 5′-D(CGCGAATTCGCG)-3′ in complex with benzimidazole derivative), 3FT6[31] (Sequence : 5′-D(CGATCG)-3′ in complex with proflavine) and 1G3X[32] (Sequence : 5′-D(CGCGAATTCGCG)-3' (DNA-3) in complex with acridine-peptide drug) were downloaded from PDB and prepared for docking using Schrödinger suite. While preparing the DNA molecules, hydrogen are added to the polar atoms and the valency of metals ions were corrected properly. The DNA molecules were minimized to some extend after adding hydrogen. Finally

the grid (with a dimension 10 Å) was prepared on the crystallographic ligands. The dimension of the grid was in such a manner that the grid will cover the complete DNA. Finally the ligands were allowed to dock on the prepared grid volume using extra precision docking method. Among the three DNAs, 443D and 3FT6 were used to dock 2,4 D and tacrine respectively. At the same time 1G3X was used for the docking studies of designed hybrids (combilexins).

**Computational designing of combilexins**

Based on the molecular docking results, the combilexins were designed by combining 2, 4 D and tacrine using computational methods with the help of a chemist and their scheme of synthesis was determined. Different carbon and polyamine linkers were used to connect both ligands. Apart from that, different functional groups and atoms were substituted by replacing two 'Cl' atoms located in the 2,4 D. While designing the hybrids, the length of the carbon and polyamine linker has been adjusted in such a manner that the designed hybrids must get a highest binding energy.

**Molecular dynamics simulation**

Based on the binding free energy, the three highest scored combilexins were selected and performed molecular dynamics simulation in order to check the residence of time of these ligands at their binding positions. The whole MD simulation studies were carried out with Desmond Software (Schrödinger suite). Prior to perform MD simulation, the DNA-drug docked complex was prepared using prepwizard and included in an orthorhombic box (dimension 10 x 10 x 10 Å and volume 132305 $Å^3$) having TIP3P water molecules. Around 18 numbers of Na+ ions and 0.15 M NaCl were also added to the orthorhombic box to neutralize the complete system. Further the MD simulation was carried out by applying OPLS force field. During MD simulation intermediate structures were saved at each time interval of 20 ps and were

superimposed to native structure to deduce the RMSD. The total time for MD simulation was 20 ns.

**Binding free energy calculation and ligand enrichment studies**

The binding free energies of known and designed combilexins were deduced and compared using prime MM-GBSA method. The method includes OPLS molecular mechanics (MM) energies ($E_{MM}$), surface generalized born salvation model for polar solvents ($G_{SGB}$) and a non polar salvation term ($G_{NP}$). The binding free energy was calculated using the following equation $DG_{binding} = G_{complex} - (G_{protein} + G_{ligand})$ where $G = E_{MM} + G_{SGB} + G_{NP}$

Since there are no experimental studies with the designed combilexins, a post docking enrichment calculation was carried out to validate the docking method. Initially a set of drug like molecules (decoys) were collected from the Schrödinger web site. The decoy set consists of 1000 drug like compounds of average molecular weight 400 Daltons. Then the active combilexin molecules were incorporated in to the decoy set and allowed to screen against the predefined grid area of the duplex DNA. Finally the performance of ligand docking and scoring was judged through receiver operating characteristics curves by checking the ability to detect only active compounds from the decoy sets.

**Results and Discussions**

From the results of ITC, it was identified that 2,4 D is perfectly interacting with DNA-1 and tacrine with DNA-2. Before performing the experiments it was predetermined that 2,4 D will titrate only against DNA-1 and tacrine against DNA-2. The predetermination was done based on the preceding reports in which it was clearly mentioned that molecules which are structurally similar to 2,4 D can interact in the minor grooves of DNA and hence the experiments were done using the duplex DNA having sequence 5′-D(CGCGAATTCGCG)-3' (DNA-1)[30,31]. Similarly the

crystal structure of acridine moiety in complex with DNA having sequence 5′-D(CGATCG)-3′ (DNA-2) was already reported[30,31]. Tacrine exhibited extreme structural similarities with the reported ligand and hence the experiments were done with DNA 2. The details of ITC analyses of both compounds were shown in Table 1. The binding isotherm (figure 1 A and B) for two compounds were found to be endothermic in nature and their stochiometry (the number of binding sites) were approximately one for both compounds. Similarly the binding affinities and the binding free energies are almost equal (-6.21 and -6.18 kcal/mol for 2,4 D and tacrine respectively) for both compounds.

The atomic level of interaction of these compounds has been predicted by molecular docking studies. As discussed earlier the DNA-1 and DNA-2 were used for both 2,4 D and tacrine respectively. The binding modes of these compounds were shown in figure 2 A and B. The glide score obtained for 2,4 D and tacrine were -6.89 and -6.41 kcal/mol respectively. From their binding modes it was observed that these two compounds are binding to the DNA through hydrogen bonds and extensive number of van der Waals contacts. In the case of 2,4 D, the carboxyl group is mediating two hydrogen bonds with two bases like guanine and cytosine. At the same time the primary amino group of tacrine is forming a hydrogen bond with one of the oxygen atom of the ribose sugar of the DNA.

The combilexins were designed with the help of a chemist and its scheme of synthesis was studied in order to confirm the synthetic viability. Mainly the modification has been done at the marked positions as shown in the figure 3. Among these, at positions, 1, 2 and 3, atoms like Cl, Br, I and F and groups like hydroxyl, amine and methyl were substituted. Similarly at positions 4 and 5 groups like amine and methyl groups were added. In positions 6-9 different carbon and amide linkers with different lengths were substituted. It was noted that substitution of methyl at

any positions (1-5) are not promising since this bulky group seriously interfere in the alignment of combilexins in the minor groove even though the other part is properly maintaining the stacking interaction. Apart from that, the substitution of amine enhances the binding affinity in majority of the designed combilexins due to electrostatic interaction (since backbone of DNA is negatively charged, there is a chance for electrostatic interaction). Similarly the substitution of hydroxyl group also enhances the interaction energy as they are involved in the hydrogen bonding. It was observed that the substitution of halogen atoms like 'Br' and 'I' enhances the binding affinity to some extend compared to the 'F' atom substitution because of the enlarged van der Waals radii of these atoms. The possibilities of halogen bond formation was also observed during the substitution of halogen atoms. During the substitution of linkers it was noted that, linkers with specific length can only exhibit a stable binding. If the linker length increases or decreases beyond 8 or 6 Å, the overall alignment of combilexin in minor groove was found to be distorted. All together 500 combilexins were designed and subjected to screen against DNA-3. Their binding affinity in terms of glide score were ranges from -5 to -13 kcal/mol respectively. Among these compounds, top scored three compounds (named as com-1 to 3) were selected and their interactions were studied in detail (Figure 4 A to C). The binding affinities of these compounds (com-1 to 3) in terms of glide score (-13.74, -12.10 and -11.89 kcal/mol respectively) and binding free energies (-76.32, -70.06 and -67.33 kcal/mol respectively) were calculated. All these compounds were exhibited a classic stacking as well as minor groove binding modes as like other combilexins does. Also several hydrogen bonds, halogen bonds, π-π interactions and various hydrophobic interactions were observed for these compounds towards DNA-3. Com-1 interacts with DNA-3 through 4 hydrogen bonds and 203 val der Waals contacts. It was observed that the keto group present in the compound exhibit a hydrogen bond with one of the 'N' atom

present in the guanine base. Similarly the secondary amines presented in the linker were found to involve in two hydrogen bondings with one of the 'N' atom of an adenine base and with 'O' atom in the sugar molecule located in the backbone of DNA. Similarly a hydrogen bond was observed between primary amine of tacrine moiety and 'O' atom of the sugar backbone of DNA. Similarly Com-2 and 3 exhibited 3 hydrogen bonds and 190 and 176 van der Waals contacts respectively. In the case of com-2, the hydroxyl and tertiary amine located in the linker is mediating two hydrogen bonds with one of the 'NH' group of guanine base and 'N' atom of adenine residue respectively. Similarly the primary amine of the tacrine moiety is mediating a hydrogen bond with one of the 'O' atom located in the sugar molecule of the backbone of DNA. In the case of com-3 all the three hydrogen bonds are raised from the linker. The imine and two amine groups located in the linker is involved in the hydrogen bonds with keto group of one of the cytosine base, 'N' atom of the adenine base and 'O' atom of one of the sugar located in the back bone of DNA respectively. All the halogen atoms are capable of producing halogen bonds with the polar atoms located in their vicinity. They are also considered to be one of the stabilizing factor in the interactions. Studies proved that the incorporation of halogen atoms in the ligand always enrich the specificity in an enzyme interaction. It also helps to fold the biological macromolecules [34]. From the binding poses of designed combilexins the possibilities of the formation of halogen bonds were investigated. In all designed combilexin molecules, halogens atoms like 'Cl' and 'Br' are presesnt. The maximum and minimum distance between halogen atoms and any of the polar atom of DNA is 3.7 and 2.3 Å respectively. So it can be stated that halogen bonding may also contribute to the binding of combilexins towards the DNA. So from the whole molecular docking studies it was revealed that designed combilexins are exhibiting more binding affinities than 2,4 D and tacrine. In order to check the efficiency of the

designed combilexins, their binding affinities were compared with already reported combilexins. All the combilexins selected for studies are having two intercalating units which are connected through a minor groove binder. These combilexins are connected to DNA mainly by hydrophobic interactions. The binding affinities (in terms of both G score and binding free energies) of already reported combilexins were deduced through molecular modeling studies and found that it is comparable with that of the designed combilexins (Table 2).

Since the expertise in the chemical synthesis of the authors is limited, only computational studies was carried out to investigate the affinity of the designed combilexins towards DNA. The residence of time of each designed combilexin for a particular time period (20 ns) was investigated through MD simulation studies. The intermediate structures were saved in every particular time intervals and superimposed to the initial structure in order to deduce the RMSD. These RMSD values in Å unit were plotted in a graph with respect to the time of simulation (Fig 5 A to C). The maximum RMSD obtained in any of the MD simulation studies is 2.1 Å. In the case of Com-1 and 2 the RMSD has been stabilized between 0.9 and 1.5 Å. But at the same time RMSD of Com-3 was found to be stabilized between 1.2 and 2.1 Å. All these results clearly focuses to the stable interactions of designed combilexins towards DNA.

Since there is no experimental proof has given for the designed combilexins, the enrichment calculation has been carried out to identify the sensitivity and specificity of the designed combilexins. The Receiver operating characteristics (ROC) curve is widely used to express the results of enrichment calculations. The area under the curve is correlating with the sensitivity and specificity. Sensitivity is defined as the ability of the docking method to detect true positives, at the same time specificity is the ability to avoid false positives. So in the study, 1000 drug like molecules were downloaded from the Schrödinger website and treated them as test. The potent

combilexins molecules that are derived from the present studies were considered as actives and fed in to the test set. Then the screening has been done with complete data set and the binding free energy was taken as the criteria to identify the sensitivity of the compounds. From the studies the ROC value (i.e the area under a ROC curve) obtained was 0.968 which indicates high sensitivity for the designed combilexins towards the duplex DNA.

**Conclusion**

In the present study, the interaction of 2,4 D and tacrine was confirmed through isothermal titration calorimetry and molecular modeling studies. The studies proposed that the binding of these two molecules are through groove binding and intercalation respectively. Later these two molecules are combined through different chemical linkers using molecular modeling tools so as to achieve the structure of combilexins. Around 500 different combilexins were designed by changing the functional groups attached to the basic scaffolds of 2,4 D and tacrine. Similarly the linker length was also adjusted in such a manner to achieve the stable interactions with the duplex DNA. The schemes of synthesis of all designed combilexin were confirmed with the help of a chemist. The designed combilexins were further screened against DNA by molecular docking methods. Top 3 scored combilexins were identified based on the binding affinities and later the same was compared with that of already reported combilexins. The structural stability and residence of time of these combilexin-DNA complexes were also investigated through a series of MD simulation studies. From the present studies it can be proposed that the top scored combilexins are potentially block the duplex DNA as other reported combilexins does. In connection with cancer treatments the combilexins are having more advantages than minor groove binders and intercalator as they have a prolonged residence of time on the DNA and due to their ability to increase the DNA melting temperature. Generally combilexins are good

inhibitors of topoisomerase. Normally combilexins exert their action through dual binding modes like groove binding and intercalation.

For the years it has been proved that targeting DNA is relatively a potent way to kill the tumor cells. Even though the clinical viability of DNA binding compounds are limited due to their genotoxicity, majority of the anti cancer chemotherapeutic drugs available in the market are DNA binding agents. Apart from these, DNA binding compounds are also used for other treatments like malaria. So DNA binding compounds have increased demand in the drug discovery field. In the current scenario groove binders and intercalators are abundant compared to the combilexins. Hence for the same reason, novel combilexin molecules with increased binding affinity and selectivity towards DNA are always welcome in order to pursue the goal of killing cancer cells with our further side effects.

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## Figure and Table legends

### Figures

**Fig 1.** Isothermal titration calorimetric analysis of 2,4 D (A) and tacrine (B) with DNA. The raw thermal power signal (top) and plot of integrated heat versus ligand/protein molar ratio (bottom) were shown.

**Fig 2.** Binding mode of 2,4 D (A) and tacrine (B) with double stranded DNA. Ligands were represented in thick lines and hydrogen bonds were represented in dashed lines.

**Fig 3.** Structures of 2,4 D and tacrine and the proposed sites for modification were highlighted in ash colour.

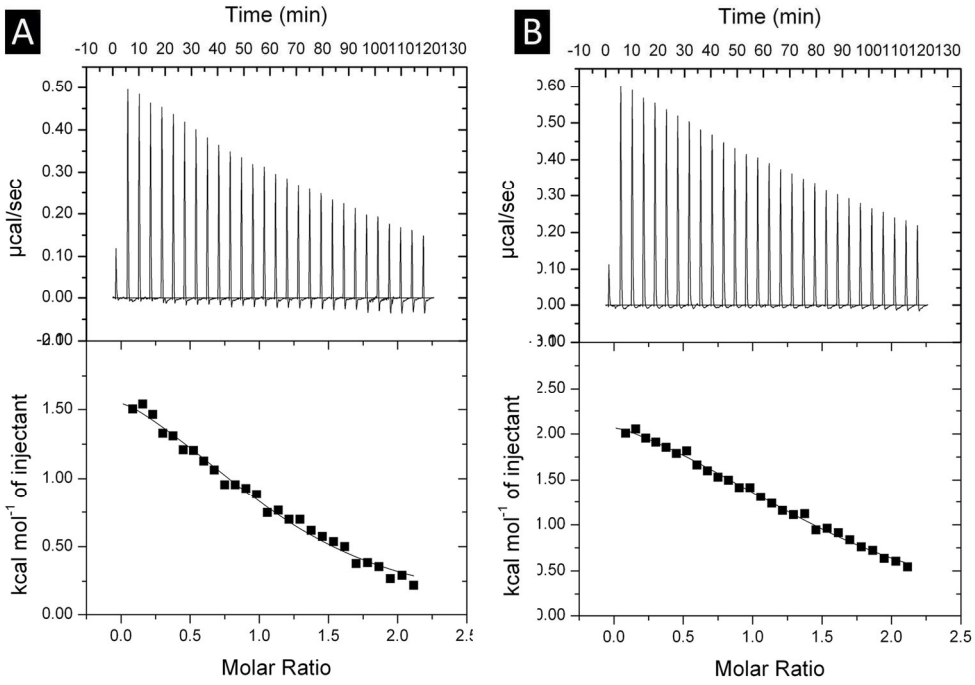
**Fig 4.** Binding mode of designed combilexins Com-1 (A), Com-2 (B) and Com-3 tacrine (C) with double stranded DNA. Ligands were represented in thick lines and hydrogen bonds were represented in dashed lines.

**Fig 5.** A plot of RMSD versus time during 20 ns MD simulations of DNA complexes with Com-1 (A) Com-2, (B) and Com-3 (C).

### Tables

**Table 1.** Thermodynamics parameters of 2,4 D and tacrine binding to duplex DNA.

**Table 2.** Comparison of binding affinities (in terms of G Score in kcal/mol) of newly designed and reported combilexins.

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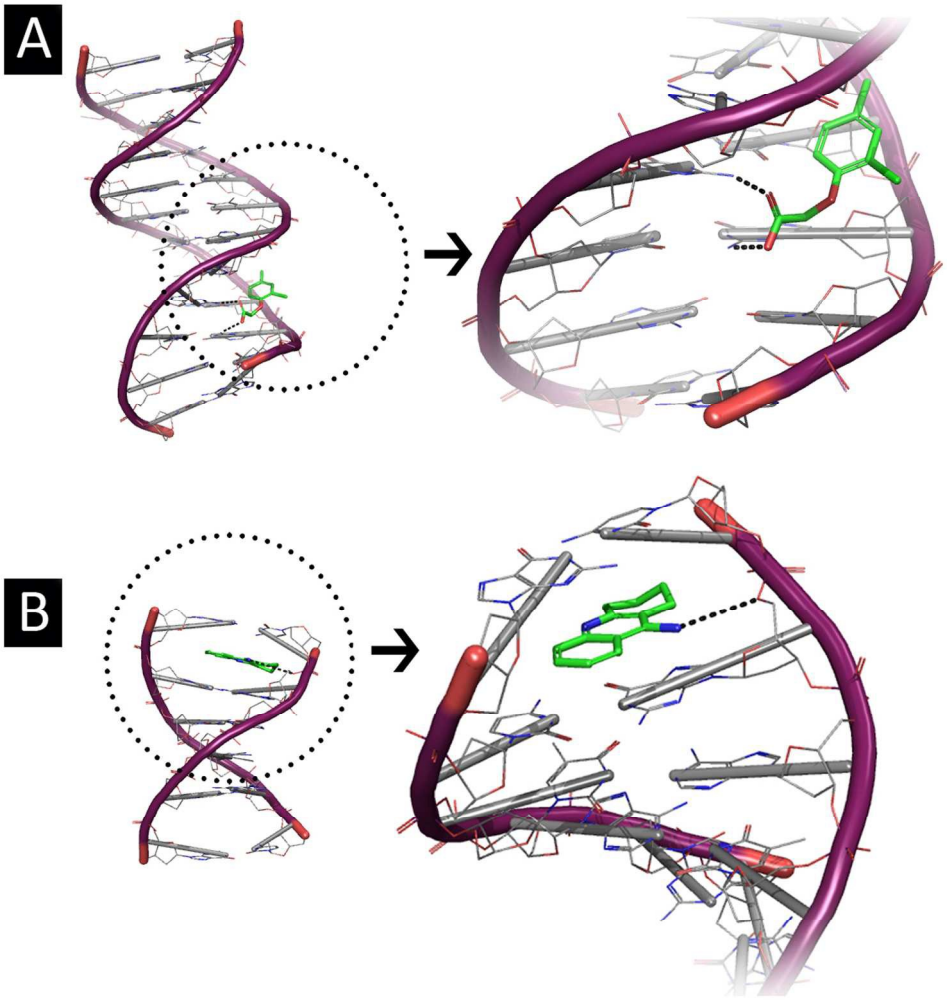


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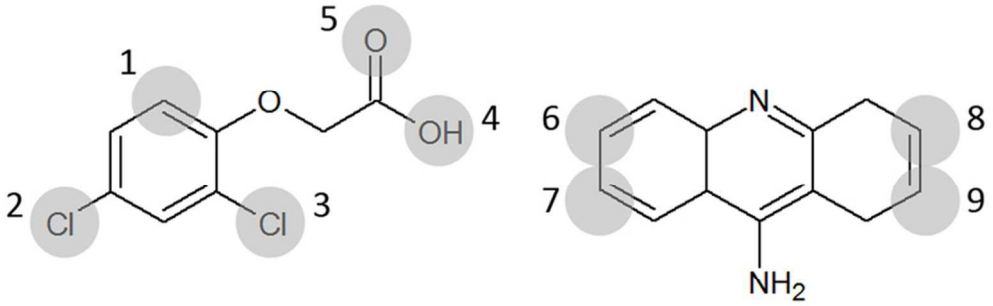
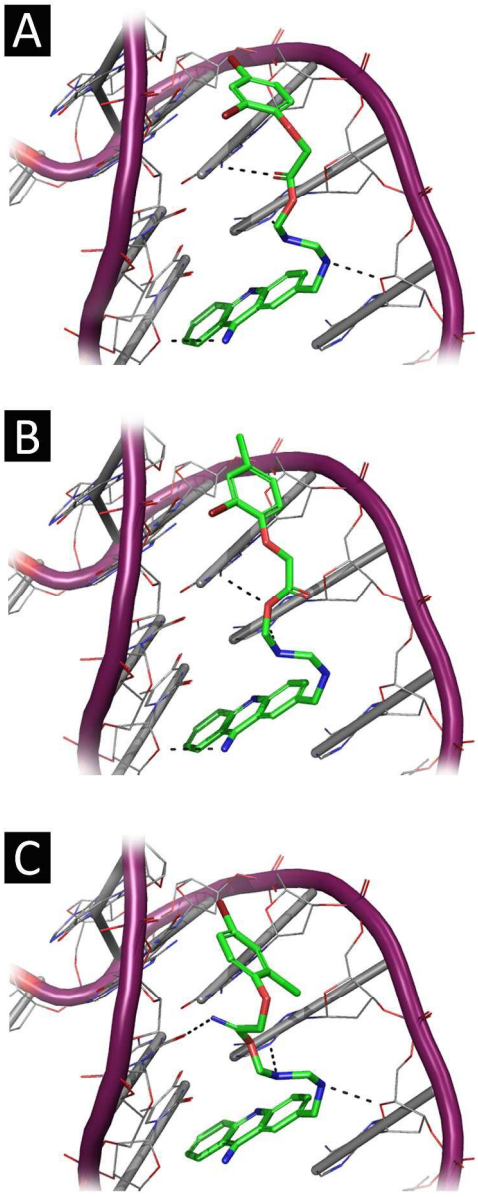
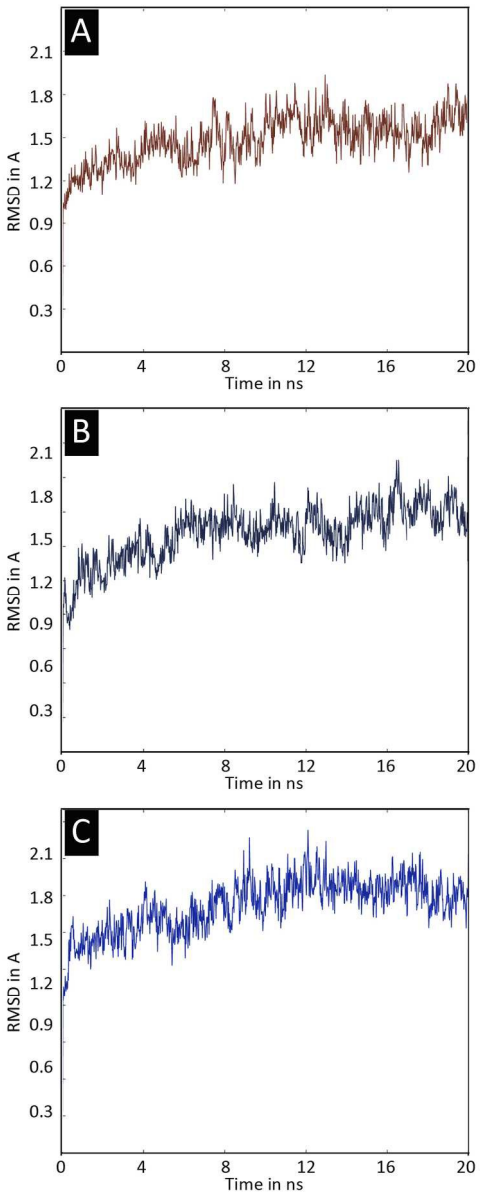



2,4 D Tacrine

77x29mm (300 x 300 DPI)




233x570mm (300 x 300 DPI)




229x567mm (300 x 300 DPI)

**Table 1**

| Sl No | Compounds | N | K in $mol^{-1}$ | T in *K* | ΔS in cal $mol^{-1}$ deg | ΔH in cal $mol^{-1}$ | ΔG in kcal mol-1 |
|---|---|---|---|---|---|---|---|
| 1 | 2,4 D | 1.16 | $3.56 \times 10^4$ | 298.15 | 27.8 | 2079 | -6.21 |
| 2 | Tacrine | 1.54 | $3.36 \times 10^4$ | 298.15 | 29.6 | 2641 | -6.18 |

**Table 2**

| No | PDB ID | Ligand Information | Binding Affinity in terms of G Score in kcal/mol | Binding free energy in kcal/mol |
|---|---|---|---|---|
| 1 | -- | Com-1 | -13.74 | -76.32 |
| 2 | -- | Com-2 | -12.10 | -70.06 |
| 3 | -- | Com-3 | -11.89 | -67.33 |
| 4 | 1AL9 | Bis-daunorubicin | -14.67 | -80.42 |
| 5 | 1AMD | Bis-daunorubicin WP-652 | -12.18 | -73.77 |
| 6 | 108D | TOTO * | -11.66 | -70.87 |
| 7 | 2MG8 | XR5944 (MLN944)** | -10.81 | -64.17 |

* 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-(3-methyl -2,3- dihydrobenzo-1,3-thiazolyl-2-methylidene)qui nolinium] tetraiodide

** 1-methyl-9-[12-(9-methylphenazin-10-ium-1- yl)-12-oxo-2,11-diaza-5,8-diazoniadodec-1-anoyl]phenazin-10-ium