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Comparative Study of dG Affinity *vs.*DNA Methylation Modulating Properties of Side Chain Derivatives of Procainamide: Insight Into Its DNA Hypomethylating Effect

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Procainamide derivatives have been synthesized to investigate the role of side chains in modulating DNA methylation level in cancer cells and gain insight into its mechanism of action. Synthesized derivatives comprises of flexible (dimethyl), constrained (pyrrolidine, piperidine, morpholine) and planar aromatic (pyridine, phenyl) side chain motifs. The affinity of Procainamide and its derivatives towards the deoxyguanosine (dG) base in neutral form has been assessed by performing Differential Pulse Voltammetry (DPV) under physiological condition. Further, molecular docking with hemimethylated CpG rich DNA acquired from active mDNMT-1-DNA (PDB ID-4DA4) crystal structure, reveals their preferential non-covalent interaction with dG nucleobase in the intercalation cavity of the minor groove. Differential affinity of the derivatives to dG base in neutral and bound form (DNA) is correlated with their DNA methylation modulating properties at sub-lethal concentration. Among all the derivatives, a compound with aromatic phenyl side chain (**1**) has shown a highest binding affinity for dG nucleobase in neutral form as well as for partially denatured CpG rich DNA which is attributed to formation of π···π stacking interaction in addition to N-H···O Hydrogen bonding with pyrimidine ring of dG base. It also shows the highest cytotoxicity and global hypomethylation at a sub-lethal level in MCF-7 cancer cell line compared to other derivatives and Procainamide. Docking study has also illustrated the plausible structural basis of DNA methylation modulating a property of Procainamide. Strong association of Procainamide with dG bases of partially denatured CpG rich DNA *via* H-bonding and other non-covalent interactions may alter the active topology of DNA required by the DNA-binding regulatory proteins (e.g. DNMT-1) which is validated by DNMT-1 inhibition assay. This systematic investigation leads to finding a new potent alternative to Procainamide and gives a plausible insight into DNA hypomethylating effect of Procainamide.

Introduction

The epigenetic regulation such as DNA methylation and histone modifications play a major role in embryonic development, cellular differentiation, suppression of repetitive elements, X-chromosome inactivation, and long-term memory.[1] Furthermore, it regulates gene expression by reversible and heritable changes in DNA without altering the nucleotide sequence of the genome. DNA methylation is a key epigenetic regulatory process that predominantly occurs in CpG rich DNA clusters called "CpG islands" present at the promoter regions of genes[2]. The process involves reversible modification of DNA by covalent addition of the methyl group at 5-carbon position of the cytidine. Cancer cells show significantly altered DNA methylation profiles compared to normal cells that are used as diagnostic and prognostic markers for many cancer types.[3] Global hypomethylation observed in cancer cells is responsible for inducing genomic instability owing to under-methylation of endogenous retroviral elements, oncogenes, and heterochromatic DNA repeats. However, aberrant hypermethylation within promoter regions of tumor suppressor genes is accountable for uncontrolled growth.[3]

The strategy of utilizing DNA hypomethylating agents (DHAs) for reducing aberrant methylation level of regulatory genes is used to treat cancer and neurological disorders.[4] Nucleoside analogs such as 5-azacytidine, 5-aza-2'-deoxycytidine, zebularine, and 5-fluro-2'-deoxycytidine have shown to reduce aberrant methylation level in cancer cells by inhibiting the activity of DNA methyltransferase (DNMT) enzymes.[5] However, their clinical side effects and genotoxicity remained a great concern. Non-nucleoside methylation modulators are the promising alternative due to their low genotoxicity and mutagenicity. The non-nucleoside DNA methylation modulators are broadly categorized into three major classes based on their site of action and binding modes. The first one is the catalytic / allosteric site (protein) binding compounds

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that interfere with the catalytic activity of the DNMTs[6] whereas the second one is CpG rich DNA binding compounds that form complex with DNA and reduces their affinity for DNMT proteins.[7] The third category of compounds compete with DNA substrate and block the DNA-binding pocket of DNMT proteins.[8] Recently FDA approved local anaesthetic drug Procaine, and antiarrhythmic drug Procainamide have been explored as DNA hypomethylating agents (DHA) against numerous malignancies as well as type-II diabetes both in *vitro* and in *vivo*.[9] A striking feature of their action is their ability to modulate DNA methylation level at sub-lethal concentration.[10] Non-genotoxicity, low cytotoxicity, and protective effect make them suitable anticancer agents for the combinatorial chemotherapeutic approach.[11] In combination with the pharmacologically active scaffold of other DNMT inhibitors or co-administration of Procaine and Procainamide with known anticancer drugs has shown higher potency against many cancer types.[12] However, the exact mechanism of their DNA methylation modulating activity so far is not clearly understood. The first report of their interaction with genomic DNA was noticed when patients undergoing prolonged therapy of Procainamide and Procaine showed the presence of antinuclear antibodies in the serum and 30% of them developed systemic lupus erythematosus syndrome.[13] Pathogenesis of Procainamide was linked to stabilization of immunogenic CpG rich Z-DNA and preferential affinity towards CpG rich DNA sequences.[14] Link between CpG rich DNA binding affinity and DNA methylation modulating property of Procainamide was first established by Lee *et al.*[15] It has been suggested that Procainamide inhibits binding of DNMT-1 to hemimethylated DNA leading to suppression of its catalytic activity. Based on these reports Procainamide was categorized as class II demethylating agent. However, recently reported docking studies[16] with constructed homology models also suggested Procainamide may form strong association with amino acids (Arg174) of DNA binding pocket of DNMT-1 protein that may reduce its affinity for 2'-deoxycytidine of target hemimethylated DNA consequently may serve as class III methylation modulator.

One way to investigate the mechanistic basis of bioactive compounds is to design derivatives and study their structure-activity relationship.[17] This approach not only provides the insight into the mechanism but also offers lead for improving the therapeutic value of the drugs. However, it is necessary to identify pharmacophore moiety within the drug molecule whose presence is essential to trigger the desired biological response. Our investigation attempts the similar approach. Evidence reported earlier for Procaine[18] has been used as clues to choose selective derivatives of Procainamide. Procaine exhibits the strong association with the guanine base compared to other nucleobases mainly due to strong hydrogen bonding between 4-amino benzoic acid backbone and guanine base. Furthermore, constrained side chain derivatives of Procaine reveal higher demethylating activity[18] compared to their flexible counterparts. On the similar note, side chain derivatives of Procainamide may also possess differential DNA hypomethylating activity if 4-aminobenzamide (anchoring site) remains unmodified.

Here we report the synthesis of Procainamide derivatives comprising flexible (dimethyl), constrained (pyrrolidine, piperidine, morpholine) and planar aromatic side chain motifs (pyridine, phenyl) without modifying 4-amino benzamide backbone. Diethyl side-chain motif of Procainamide is also flexible due to free rotation of both the ethyl moieties covalently linked to tertiary nitrogen. The differential affinity of Procainamide and its derivatives towards dG nucleobase in the neutral and bound state have been investigated using DPV and molecular docking study respectively. Further, we explored growth inhibitory properties of these compounds towards MCF-7 cancer cell lines, quantified global DNA methylation level at a sub-lethal concentration and their effect on DNMT1 activity *in vitro* to evaluate cytotoxicity and suitability as DHA. It can be seen that the differences in the affinity towards dG nucleobase and CpG rich DNA, cytotoxic effect, global hypomethylation at sub-lethal level (MCF-7 cancer cell line) are correlated with the structural features of side chain motifs. This comparative study has helped in deriving the relation between dG affinity and DNA methylation modulating activity of Procainamide and its derivatives. Based on the study we have also hypothesized the plausible mechanism by which Procainamide may act as DHA in cancer cells.

Result and Discussion

Chemistry

Procainamide derivatives were prepared as per the reported procedure (Figure S1, ESI).[19] 4-nitro benzoyl chloride was added to primary amines in the presence of triethylamine base to form corresponding 4-nitro carboxamides. Further reduction of the nitro group in the presence of catalytic amount of Pd/C and H_2 yielded compounds **1-6**. The compounds were named based on their side chain. They were further characterized by ^{1}H NMR and HR-MS (Figures S2-S13, ESI). Good quality crystals were grown for single crystal X-ray diffraction. The Purity of the compounds was checked by HPLC (Figures S14-S19, ESI) and further used for biological studies.

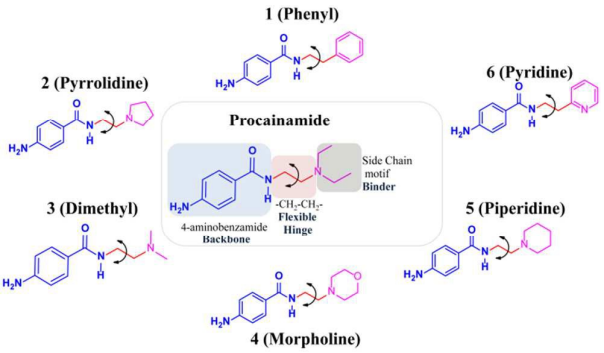

Figure 1. Description of pharmacophore features of Procainamide and molecular structures of its side chain motif derivatives **1-6**.

Crystallography

Molecular conformations in crystal structures and solution state share some analogy. Comparative analysis of crystal structures of isostructural compounds provides valuable information about their preferred geometry and torsional constraints of rotatable bonds within pharmacophore.[20] Pure compounds were crystallized from dichloromethane/ petroleum ether solvent mixture by a slow evaporation method. Crystal structure analysis was carried out using Bruker Smart Apex II X-ray diffractometer. Detailed information about crystallographic data and ORTEPs diagrams are provided in ESI (Table S1, Figure S20, ESI). Atomic coordinates of Procainamide were retrieved from Cambridge Structural Database (CSD, CCDC number 694545).[21] Based on reported studies with Procaine[18] the 4-aminobenzamide of Procainamide may play a pivotal role as backbone moiety of pharmacophore.[18] Similarly alkyl chain (-CH_2-CH_2- hinge) may play a significant role in providing flexibility to side chains (binder) (Figure 1)and eventually responsible for the structural variation. Structure overlay (Figure 2a) and torsion angle τ_1 (C1-C7-N-C8), τ_2 (C7-N-C8-C8), and τ_3 (N-C8-C9-R) are tabulated in (Figure 2b, Table 1). As expected the torsional difference across 4-aminobenzamide backbone showed less change whereas torsional variation across side-chains displayed marked deviation.

Electrochemical studies

Electroactivity of nucleobases has been used for their quantification in biological samples as well as to study their interaction with other chemical species.[22] DPV study and DFT calculation reported earlier[18] for Procaine and DNA bases reveal that binding affinity of Procaine towards DNA bases is in the order G > A > T > C. The strongest binding affinity for guanine base in neutral form is attributed to co-planar C-H···N and N-H···O hydrogen bonding between 4-amino benzoic acid of Procaine and pyrimidine moiety of guanine. Similarly,

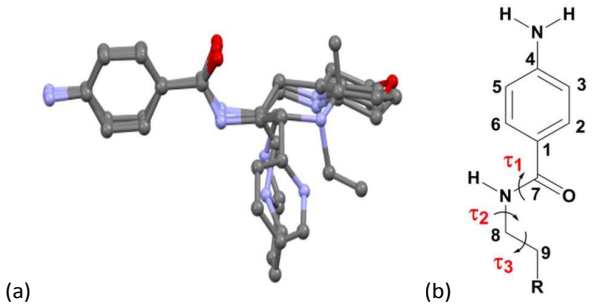

Figure 2.(a) Structure overlay of Procainamide and its derivatives and **(b)** scheme displaying different sites of torsion in the compounds.

Compounds	τ_1(°)	τ_2(°)	τ_3(°)
Phenyl(1)	171.71	94.6	179.08
Pyrrolidine(2)	172.68	-168.93	-51.77
Dimethyl(3)	-175.34	-139.39	-61.58
Morpholine(4)	-172.43	-101.03	-164.72
Piperidine(5)	-170.0	-105.96	-167.33
Pyridine(6)	173.80	84.44	71.66
Procainamide	166.14	88.18	173.24

Table 1. Torsion angles (τ_1–τ_3) of compounds **1-6** and Procainamide extracted from their crystal structures.

Procainamide may also exhibit the strong association with guanine due to the presence of 4-aminobenzamide moiety. The binding affinity for cytosine base was found to be much lower than other bases. CpG rich DNA is built by the alternate arrangement of guanine and cytosine bases linked *via* phosphate-deoxyribose backbone. In view of this, binding affinity towards guanine was employed as a parameter for structure-activity correlation. We envisaged that the affinity study with deoxyguanosine (dG) instead of guanine base may give broader view and significant variation for deriving structure-activity relation based on the fact that guanine conjugated with deoxyribose sugar residue provides multiple interaction sites. Redox electrochemical behaviour of guanine is depicted in figure 3. To investigate differential affinity of Procainamide and its derivatives (**1**-**6**) to dG nucleobase base in a neutral form, DPV was performed using CHI 900b potentiostat with a pulse amplitude of 50 mV, pulse width of 0.2 s and a pulse period of 0.5s (for detailed procedure see page S28, ESI). Figure 4 shows the differential pulse voltammograms of pure dG as well as dG in the presence of Procainamide and compounds **1-6**. When the potential of the GC electrode, kept in a solution of dG, is made more positive as compared to the open circuit potential (OCP), a clear peak is observed at 0.79 V (vs SCE, 0.1 M KCl) (there is no peak in case of buffer solution alone, Figure. S21) due to the oxidation of

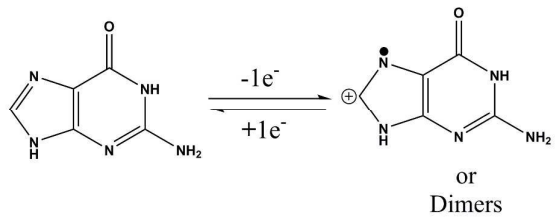

Figure 3. Scheme displaying redox behaviour of guanine base.

dG as shown in the reaction mechanism in figure 3. Moreover, when the same concentration of dG is used in all the experiments (dG to compound ratio: 4), the peak current density of dG oxidation drops in the presence of carboxamide ligands (**1-6** and Procainamide) (Figure 4). The results indicate binding of added ligands to dG and hence number of free dG molecules depletes in the solution. As a result, peak current decreases because the current is directly proportional to the concentration of the active species in the solution. It invariably suggests that the drop in peak current of dG is due to the interactions of the Procainamide and compounds **1-6** with dG. However, the extent of the drop in peak current is differentially related to structural features of side-chain motifs of ligands. Phenyl substituent is found to cause a maximum drop in peak current of dG (Figure 4b) and thus more efficiently binds to dG. The effective binding affinity of Procainamide derivatives (according to Figure 4b) follow the order, phenyl (**1**) > pyridine (**6**) ≈ piperidine (**5**) > morpholine (**4**) > Procainamide > dimethyl (**3**) ≈ pyrrolidine (**2**). The somewhat broad peak observed prior to the dG peak is due to the oxidation of compound **1-6** and Procainamide that is demonstrated by control experiment for Phenyl(**1**)(Figure S21). A broad peak observed at 0.6 V (vs. SCE, 0.1 M KCl) prior to dG oxidation peak, attributed to the oxidation of phenyl (**1**) to form radical cation. The peak that appears after dG peak corresponds the oxidative decomposition/cleavage of the compound-dG complex on the application of a more positive potential. It is supported by the fact that both dG, as well as phenyl derivative alone, do not show such peak after the oxidation potential of dG. Such peak is observed only in case of mixed solutions of dG and compound under study. Careful observation of these peaks reveals that the phenyl-dG complex requires much higher potential than any other compound-dG complex and thereby it is most stable. This result indirectly indicates the possibility of strong binding of phenyl with dG.

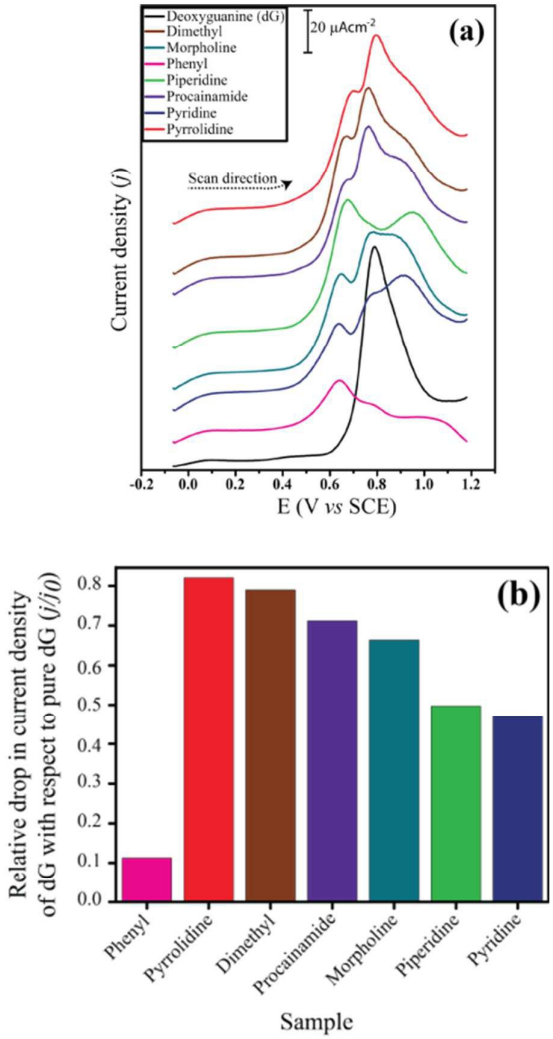

Figure 4. (a) Differential pulse voltammograms of dG in the presence of compounds **1-6** and Procainamide; glassy carbon, Pt-foil and standard calomel electrode (SCE, 0.1 M KCl) are used as working, counter and reference electrodes respectively (pulse amplitude of 50mV, pulse width of 0.2s and a pulse period of 0.5s); systematic drop in peak current density (j) in presence of different compounds suggest their differential binding affinity to dG; (b) relative drop in peak current density (j) of dG with respect to peak current of pure dG (j_0) (j is normalized with respect to j_0).

Molecular docking study

Many experimental evidences were reported for association of Procainamide with CpG rich DNA. However, two reports provided important clues about their selective affinity. First report revealed shift in the midpoint of transition is observed during salt-induced B-Z transition of poly CpG DNA in the presence of Procainamide[14] while the second report suggested it shows specific affinity for hemimethylated-DNMT-1 bound complex over unmethylated-DNMT-1 bound counterpart.[15] Cellular processes that involve dynamic topological variation of DNA such as maintenance methylation, B-Z transition, replication, DNA modification and repair share common structural features. Partial denaturation of DNA and flipping out of bases at the target site is observed during these events.[23] We predicted Procainamide may show more affinity for partially denatured CpG rich DNA and driving force is provided by its affinity for the dG base. To validate our hypothesis we used molecular docking study.

Docking study provides a good estimate of sequence specificity and intercalation mechanism of DNA-binding drugs.[24] Due to the significant variation observed for minor groove size within the crystal structures of AT-rich and GC-rich DNA sequences,[25]it is advisable to use crystal coordinates instead of modelled DNA for carrying out docking study with minor groove binding agents. The atomic coordinates of CpG rich hemimethylated DNA (hmDNA) were extracted from RCSB Protein Data Bank (PDB ID:4DA4) and used for docking study.[26] Structural analysis reveals that the H-bonding donor/acceptor groups of dG bases are exposed by partial denaturation of DNA. Further, non-conventional pyrimidine: pyrimidine base

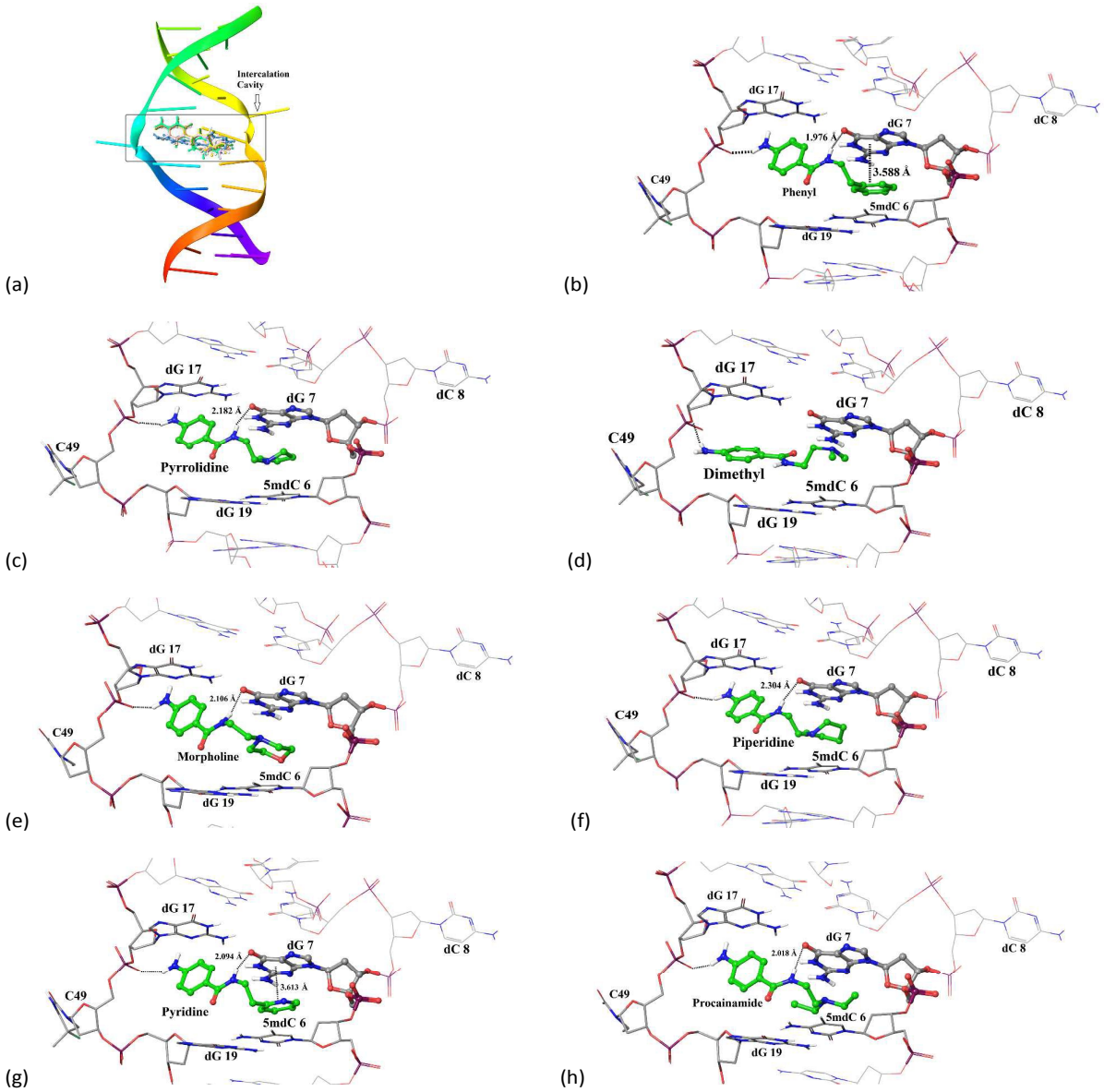

Figure 5. (a) Compilation of docked poses showed most favourable binding site lies within intercalation cavity. Detailed view of individual docked poses of **(b)** phenyl **(c)** pyrrolidine **(d)** dimethyl **(e)** morpholine **(f)** piperidine **(g)** pyridine **(h)** Procainamide with hmDNA, showing strong hydrogen-bonding association with dG nucleobase(except dimethyl) and O-acceptor of phosphodiester linkage at the target site .

pairing between dG bases and flipping out of nucleobases creates small intercalation cavity at the target site.

Docking was performed with Schrodinger Maestro Suite and flexible docking method.[27] Detailed procedure and docking protocol are provided in electronic supplementary information (pgS29, ESI). Post-docking minimization and residue-wise interaction parameters were calculated. Most important step for performing docking simulation is to generate all possible bioactive conformers of the compounds and to choose reliable docked poses for comparative analysis. Atomic coordinates of compounds **1-6** and CSD database survey[28] (Figure S22, Table S2, ESI) suggested permissible variation for torsion angle τ_1 that lies within the range of 160°-180°. Accordingly the filtering of the docked poses has been carried out where ligand conformation matched with our cut-off value. Further, conformers with lowest glide scores were used for comparative studies. Structural overlay and torsional parameters of ligand conformations extracted from docked poses are provided in ESI (Figure S23, Table S3).

Docking analysis with hmDNA reveals that all the compounds are docked inside the intercalation cavity of the DNA minor groove (Figure 5a) and have formed a strong association with a

dG base of target site except the dimethyl derivative. Carboxamide N-H proton of 4-aminobenzamide backbone formed strong N-H···O hydrogen bonding interaction with the carbonyl oxygen C=O of dG base whereas N-H proton of para-amino group formed a N-H···O hydrogen bonding with O acceptor atom of phosphodiester linkage of DNA (Figure 5b-h). Although all the derivatives except the dimethyl (**3**) showed similar binding features, variation in their H-bonding geometries with the dG base was noticed (Figure 6a). Glide score (docking score) was used to evaluate binding affinity for entire hmDNA whereas, H-bonding interaction length, H-bonding energy and van der Waals energy were taken into account for assessing the binding affinity for dG at the target site. Based on the glide score, affinity trend for hmDNA was found to be in the order, phenyl (**1**) > pyridine (**6**) > pyrrolidine (**2**) > piperidine (**5**) > morpholine (**4**) > Procainamide > dimethyl (**3**). H-bonding energy score (E1) between ligands and dG residue revealed phenyl (**1**), Procainamide, and pyridine (**6**) derivatives form strong hydrogen bonding interaction with dG nucleobase (Figure 6a). Moreover, van der Waals interaction energy scores (E2) suggests that all the derivatives except dimethyl (**5**) are strongly associated *via* van der Waals forces with dG nucleobase in addition to their H-bonding interactions. Docked postures of phenyl and pyridine showed the formation of parallel displaced π-stacking interaction between benzene/pyridine moieties with pyrimidine ring of the dG base. However, the geometrical parameters for stacking interactions (Cg···Cg distance for phenyl 3.588 Å and pyridine 3.613 Å, the dihedral angle between aromatic rings α for phenyl 15.85^{o} and for pyridine 18.67^{o}) suggests benzene ring of phenyl (**1**) formed comparatively stronger π···π interaction than pyridine ring of compound **6** (Figure 6b, S24, ESI)**.**

The molecular docking study reveals that the side-chain topology of compounds play a pivotal role for binding to CpG rich hmDNA. The Strength of the binding affinity increases as the rigidity and bulkiness of side chain motif increases. The comparative analysis showed that hydrogen bonding between 4-aminobenzamide backbone and dG nucleobase in the intercalation cavity allowed all the derivatives to dock inside the minor groove intercalation cavity but the strength of their association within target site is further determined by other non-covalent interactions between side chain and target base. The aromatic side chain phenyl (**1**) showed the highest affinity due to the formation of π···π stacking interaction with the dG base (Figure 6b).

Cytotoxicity Study

Cytotoxicity towards MCF-7 breast cancer cell lines was investigated to evaluate the differential cytotoxic effect of Procainamide and its derivatives (**1**-**6**). Five different concentrations of compounds from 500μM to 100μM at 24h, 48h, and 72h time points were used to treat the cell line. Detailed procedure and protocol are provided in electronic supplementary information (pg S32). The results depicted in electronic supplementary information showed that all the derivatives (**1**-**6**) and Procainamide were non-cytotoxic at 24h and 48h time points (Figure S25-26, ESI). Notably, even after 72h and 100μM concentration of compounds did not exhibit obvious cytotoxicity and viability ranges from 80% to 100% (Figure S27, ESI). However, at 500 μM concentration and 72h time point, significant variation in their cytotoxicity was observed (Figure 7). Phenyl (**1)** showed almost 50 % cell death with the highest cytotoxicity whereas pyridine (**6**) and dimethyl (**3**) derivatives possess moderate cytotoxicity. Procainamide, pyrrolidine (**2**), piperidine (**5**), and morpholine (**4**) containing derivatives showed the absence of significant cell death even at the highest concentration. The trend observed for growth inhibitory properties towards breast cancer cell line is in the order, phenyl (**1**) > dimethyl (**3**) > pyridine (**6**) > pyrrolidine (**2**) > piperidine (**5**) > morpholine (**4**) > Procainamide.

Global Methylation Quantification

(a)

Compounds	Glide Score	Affinity towards dG		
		H-bond Distance(Å)	E1	E2
Phenyl (1)	-8.33	1.976	-1	-7.618
Pyrrolidine (2)	-7.839	2.182	-0.795	-8.242
Dimethyl (3)	-4.254	-------	-------	-------
Morpholine (4)	-7.72	2.106	-0.987	-8.787
Piperidine (5)	-7.796	2.304	-0.434	-8.245
Pyridine (6)	-8.182	2.094	-1	-8.388
Procainamide	-6.769	2.018	-1	-7.506

(b)

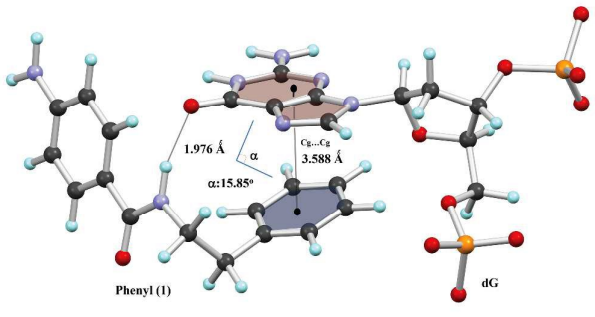

Figure 6. (a) Tabulated glide score, affinity parameters to dG residue (H-bond distance, hydrogen bond score E1, van der Waals association energy score E2 of docked poses of Procainamide and derivatives (**1**-**6**) with hmDNA and **(b)** strctural depiction of association between phenyl (**1**) and dG base at the target site *via* H-bonding and aromatic π···π stacking interaction.(Information of the terms 'E1' and 'E2' scores are provided in ESI)

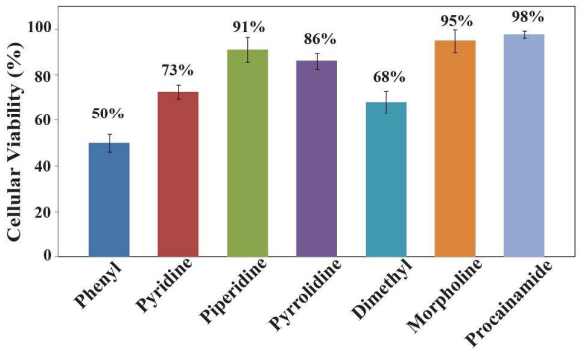

Figure 7. A comparison of cell viability assay (data in %) after 72h of treatment with 500 μM conc. of Procainamide and its derivatives (**1**-**6**) towards MCF-7 cancer cell line.

Procainamide and its derivatives (**1**-**6**) showed variation in their affinity towards the dG base in neutral form, partially

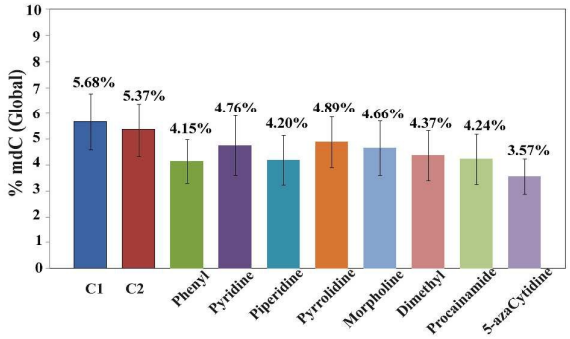

Figure 8. A comparison of global methylation quantification (in %) after 72h of treatment with 100 μM conc. of Procainamide, its derivatives (**1**-**6**) and controls (C1= blank, C2= 0.5% DMSO) towards MCF-7 cancer cell lines.

denatured CpG rich DNA and cytotoxicity towards breast cancer cell line. However, their cytotoxic effect alone may not infer their ability to modulate DNA methylation level and candidature as DHA. Secondly, Procainamide is promising combinatorial drug that reduces aberrant methylation at the sub-lethal level and increases vulnerability of cancer cells for strong anticancer drugs.[11,12] Relative shift in the global methylation level in MCF-7 cancer cells upon treatment with sub-lethal concentration of compounds is used as a parameter for structure-activity correlation. In recent years, many techniques and methods have been reported for quantifying global methylation levels. These methods are luminometric, electrophoretic, pyro-sequencing, HPLC and colorimetric-based assays.[29] For our study ELISA based methylated DNA quantification kit (colorimetric) from Abcam was used for determination of % 5-methyl-2'-deoxycytidine content (%mdC) in treated and untreated MCF-7 cancer cells. The detailed experimental procedure is provided in electronic supplementary information (Fig S36, ESI). The C1 and C2 were the two negative controls used for this study. Control C2 was used as 0.5% DMSO to eliminate the error of demethylating effect of DMSO solvent.[30] 5-azacytidine (5 μM) was used as a positive control. Methylation level in case of negative control DNA samples, C1 (no DMSO) and C2 (0.5% DMSO) ranged from 5.37% to 5.68% which is similar to one reported in published literature.[31] Positive control 5-azacytidine showed highest decrease in global methylation level whereas trend in the decrease in methylation level for Procainamide and its derivatives (**1**-**6**) followed the order as phenyl (**1**) > piperidine

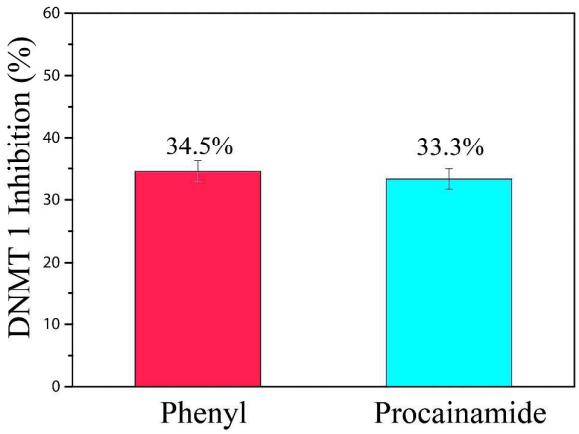

Figure 9. A comparison of *in vitro* DNMT-1 inhibitory activity (%) of compound 1(phenyl) and Procainamide at 100 μM conc. (DMSO 0.01%)

(**5**) > Procainamide > dimethyl (**3**) > morpholine (**4**) ≈ pyridine (**6**) > pyrrolidine (**2**) (Figure 8). The highest decrease in the methylation level was observed for phenyl (**1**) containing derivative whereas the lowest decrease was seen for the pyrrolidine (**2**) derivative. Previous reports have shown that slight modulation in global methylation level is attributed to significant change at the gene regulation level,[32] which suggested phenyl derivative can be used as DHA at sub-lethal concentration.

DNMT-1 Inhibition assay

DNMT-1 inhibitory activity of compound **1** (comparatively higher active) and Procainamide were assessed using DNA methyltransferase 1 activity/inhibitor screening assay core kit (P-3006A). Detailed procedure is provided in the ESI (page 37). Both the compounds showed inhibition of DNMT-1 activity in *in vitro*. However, no significant difference was observed which may be attributed to their similar mode of action (figure 9).

Conclusions

Four activity parameters namely, dG affinity (neutral state), CpG rich binding affinity, cytotoxicity to MCF-7 cancer cell

lines, % mdC level (global) showed significant variation with respect to topology of side-chain motifs of Procainamide and its derivatives. DPV study revealed that aromatic side chains containing, phenyl (**1**) and pyridine (**6**) derivatives showed strong association with the dG base in a neutral form whereas flexible side chain containing, dimethyl (**3**) and Procainamide derivatives showed the least association. Constrained

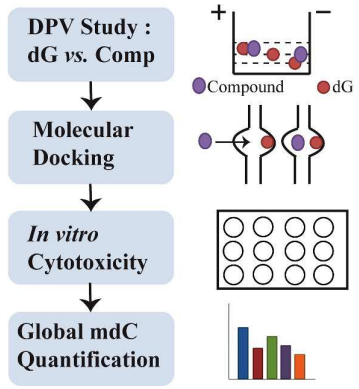

Figure 10. Cartoon illustration of protocol for the rapid method of screening for non-nucleosidal CpG DNA binding methylation modulators.

heterocyclic side chain containing morpholine (**4**) and piperidine (**5**) derivatives showed intermediate association with dG base except pyrrolidine (**2**). DPV results demonstrated good correlation with docking study with partially denatured hmDNA. Phenyl (**1**) and pyridine (**6**) derivatives formed π···π stacking interaction with the dG base that manifested their strong association with CpG rich hmDNA. Conversely, flexible dimethyl (**3**) and Procainamide showed the least affinity. Based on the glide score, constrained heterocyclic side chain derivatives possessed intermediate (between aromatic and flexible side chain derivatives) binding affinity. Combined results signify that the aromatic side chains possess a higher affinity towards CpG rich DNA due to their strong intermolecular association with dG nucleobase.

Cytotoxicity data demonstrated good agreement with DPV/docking study. It is further used to evaluate the sub-lethal concentration of Procainamide and derivatives **1-6**. Interestingly all the derivatives of Procainamide have shown the decrease in %mdC level in comparison with control in MCF-7 cancer cells at the sub-lethal concentration which may be attributed to their affinity towards CpG rich DNA especially to dG base. Moreover, phenyl (**1**) showed a higher demethylating effect compared to other derivatives (**2**-**6**) including its parent analogue Procainamide. The increased demethylating property is attributed to its strong association with dG nucleobase *via* hydrogen bonding and π···π stacking interaction within the CpG rich minor groove of DNA. Procainamide has been shown to induce structural changes in circular supercoiled plasmid DNA and alter tertiary topology of DNA.[14] On the similar note strong association of phenyl (**1**) with CpG rich DNA may alter its active conformation required by regulatory proteins such as DNMT-1 and may consequently inhibit protein-DNA binding which is validated by *in vitro* DNMT-1 inhibition assay. Thus, our study provides mechanistic insight into the plausible mode of methylation modulating activity of Procainamide and its analogues. Our systematic investigation also provides a rapid method of screening for finding other non-nucleoside DNA binding (CpG rich) demethylating agents as shown in figure 10. The screening may involve four primary steps before going to *in vivo* trial. Step one will involve the rapid evaluation of binding affinity of compounds for a dG base in a neutral form. The extent of affinity will be determined by the drop in the peak current. Step two comprise of docking simulation study with hmDNA to evaluate the affinity to partially denatured CpG rich DNA. The third step will evaluate cytotoxicity and sub-lethal concentration at *in vitro*. Finally increase or decrease in the global methylation level in cancer cells will decide the candidature of the compounds as DHA. Currently in our lab we are exploring sulphonamide based novel methylation modulators due to the additional conformational flexibility of 4-amino benzene sulfonamide compare to 4-amino benzamide backbone.

Experimental

Experimental details of compounds characterization, crystallographic, electrochemical, docking, cytotoxicity, global methylation quantification study and DNMT-1 inhibition assay are provided in electronic supplementary information file (ESI).

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Table of Content

DNA hypomethylating property of Procainamide and its derivatives is attributed to their association with the dG nucleobase of partially denatured CpG rich DNA which further reduces their affinity for regulatory proteins such as DNMT-1. Phenyl side chain derivative of Procainamide caused the highest drop in global % mdC level owing to its strongest binding affinity with the dG base.