Anticancer activity of HMGA1 promoter targeting triplex forming oligonucleotide in HeLa cell line

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High mobility group protein A1 (HMGA1) acts as an architectural transcription factor and regulates transcription of various genes. Upregulation of HMGA1 has been described in a large number of human malignancies and serves as a ‘tumor marker’. Due to its role in neoplastic transformation and tumor progression, \textit{hmga1} is considered as a promising therapeutic target. In the present study, we investigated the interaction of triplex forming oligonucleotide (TFO) of 18 bps targeted to \textit{hmga1} promoter (−1917 to −1940) and its influence on the expression of HMGA1 in HeLa cells. Stability of DNA triplex was characterized using various biophysical and thermodynamic methods and was confirmed by gel retardation assay using γ-32P [ATP]. Treatment of HeLa cells with \textit{hmga1} specific TFO significantly downregulated HMGA1 expression at mRNA, protein levels (~48%) and inhibited cell proliferation as investigated by RT-PCR, Western blot and Flow cytometry. The findings of the study suggest that TFO-mediated inhibition of \textit{hmga1} expression can be a promising strategy for modulation of gene expression and for inhibition of cancer cell proliferation. Moreover, DNA triplex-based therapeutic approaches hold promise in combating cancers associated with HMGA1 overexpression.

Keywords: Cancer, DNA triplex, Energetic, HMGA1 expression, Triplex forming oligonucleotide

High mobility group A1 (HMGA1) is a member of the superfamily of non-histone chromosomal proteins, and is known as an ‘architectural’ transcription factor that regulates transcription of various genes\textsuperscript{1,2}. The HMGA group was initially discovered in proliferating human cervical cancer HeLa S3 cells by Lund and his colleagues\textsuperscript{3}. Since their initial discovery, HMGA1 group captured a lot of attention because of their oncogenic properties\textsuperscript{4}. HMGA1 is characterized by ‘AT-hook’ DNA binding motif which binds to DNA leading to recruitment of various transcription factors. This facilitates the formation of nucleoprotein complex, called ‘enhanceosome’, which is involved in transcription of various genes\textsuperscript{5}. Increasing evidence suggests that HMGA1 protein functions as a master regulator with a critical role in normal development and tumor progression in diverse malignancies\textsuperscript{6,7}. Several studies demonstrated that HMGA1 protein has a critical role in tumor initiation\textsuperscript{8,9}, and its overexpression is mainly associated with human malignant neoplasias, including thyroid\textsuperscript{10}, colon\textsuperscript{11}, skin\textsuperscript{12}, and breast\textsuperscript{13} carcinomas, etc. We have earlier shown that HMGA1 expression is high in proliferating tissues like testes and thymus, while low or undetectable in liver (with a low fraction of cells undergoing division) using animal model\textsuperscript{14}. Shah & Resar\textsuperscript{5} recommend HMGA1 to be a promising candidate ‘biomarker’ and therapeutic target in cancer. The oncogenic effects of HMGA1 are likely related to its ability to activate expression of various genes that promote cell proliferation and invasion or prevent apoptotic cell death. Therefore, targeting architectural transcription factor may be promising therapeutic strategy for various cancers associated with overexpression of HMGA1.

Therapies targeted against HMGA1 have been widely anticipated as a potential antitumor strategy. Therefore, taking account of roles of HMGA1 in human malignancies, different approaches are being attempted to downregulate the expression or to inactivate HMGA1 protein. Synthetic molecules like netropsin, berenil and FR900482 were used to block HMGA1 protein from binding to their cognitive gene promoters and drugs like lexitropsins were attempted to inhibit the promoter regions of genes that are regulated by HMGA1 protein\textsuperscript{15}. The above mentioned attempts targeting HMGA1 at translational level are found to be partially and non-specific in nature. Therefore,
antigene or antisense based strategies create themselves as the most specific and effectual approach as compared to them. There are various means by which antigene strategy has been attempted in context to \textit{hmga1} in diverse array of cancer cell lines\textsuperscript{16,17}.

Triplex forming oligonucleotide (TFO) binds with high affinity and specificity to purine rich sequences of double stranded DNA. TFO represents an attractive tool for manipulating gene sequence and expression\textsuperscript{18}. Intermolecular triplex formation requires a purine rich stretch of duplex DNA to which a single stranded TFO can bind through Hoogsteen hydrogen bonding in either a parallel and anti-parallel orientation. Anti-parallel triplexes are typically formed by purine (GA) or (GT) TFOs forming canonical G*G-C, A*A-T and T*A-T triplets (* denotes Hoogsteen base pairing while, – indicates Watson-Crick base pairing) through reverse Hoogsteen hydrogen bonding. Parallel triplexes are formed by pyrimidine TFOs forming canonical C+*G-C (C+ denotes protonation of N3 of C) and T*A-T triplets resulting from Hoogsteen hydrogen bonding\textsuperscript{19}. However, protonation of N3 in C requires a low pH below physiological condition (i.e., pH<5) leading to its limitation in the application of unmodified pyrimidine TFOs in cells. Morgan and Wells first demonstrated promising biological role of triplex structure in 1968\textsuperscript{20}. Various reports demonstrated TFO-mediated activities in cell culture experiments\textsuperscript{21-24}. We have already shown that \textit{c-met} specific DNA triplex based therapeutic approach is a promising tool in the treatment of malignancies associated with MET overexpression\textsuperscript{25}.

In this study, we report \textit{in-vitro} formation and stability of DNA triplex using various biophysical techniques and assessment of expression level of HMGA1 in response to \textit{hmgal}-TFO in HeLa cells. We employed antigene strategy to target \textit{hmgal} and our findings suggest that TFO is a potential therapeutic alternative in combating cancers.

**Materials and Methods**

**Reagents**

Single stranded oligodeoxyribonucleotides 3'-CCCGAGAGAGGGCGGACAGGAC-5' and its complementary 5'-GGGCTCTCTCTCCCGCCTGTCCTG-3' from \textit{hmgal} gene promoter (~1917 to ~1940) were used for DNA duplex formation. The sequences of designed \textit{hmgal}-TFO were 5'-GAGAGAGAGGGGGGAGAG-3', while the control TFO GGGAGGGAAGAGAGAGAA, had the same length with mismatched (one base per codon) at different positions of \textit{hmgal}-TFO. The phosphorothioated oligodeoxyribose nucleotides were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Sodium cacodylate was purchased from Fluka (Fluka Chemical Corp., USA). All other reagents used for the experiments were of analytical grade. The concentration of oligodeoxyribonucleotides was determined spectrophotometrically using the molar extinction coefficients which were calculated by using nearest neighbor analysis of the unfolded species\textsuperscript{26}. Unless otherwise indicated, all biophysical experiments were performed in 10 mM Sodium cacodylate buffer containing 100 mM NaCl and 10 mM MgCl\textsubscript{2} at pH 7.0. All buffer solutions were passed through a Millipore filter of 0.45 µm to remove any particulate matter.

Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal calf serum (FCS), Trypsin-EDTA, penicillin G, streptomycin and amphotericin B were obtained from Gibco (MD, USA). Lipofectamine\textsuperscript{TM} LTX Reagent (Cat. No. 15338-100) along with PLUS\textsuperscript{TM} Reagent (Cat. No. 11514-015) used for the transfection procedure, were procured from Invitrogen, CA, USA. The TRIzol reagent of Invitrogen was used for RNA isolation. For reverse transcription of total RNA, miScript II RT Kit (Cat no: 218161) of Qiagen (Valencia, CA, USA) was used. Antibodies, anti-HMGA1 and anti-β ACTIN were procured from Sigma-Aldrich Corporation (St. Louis, MO, USA). All other chemicals used for experiments were of analytical grade. The radioactive material, $\gamma$-32P[ATP] was procured from Bhabha Atomic Research Center (BARC), Mumbai and T4-polynucleotide kinase and kinase buffer were obtained from New England Biolabs.

**Identification of triplex target sequences (TTSs)**

Triplex forming oligonucleotide target sequence search tool was used to search TTSs in \textit{hmgal} promoter\textsuperscript{27}. The TTS search parameters were set with minimum of TTS length of 15-nt, minimum percent G composition of 50%, and allowed 3 pyrimidine interruptions.

**Circular Dichroism (CD) spectral study**

All Circular Dichroism (CD) experiments were performed on a JASCO-810 spectropolarimeter equipped with inbuilt Peltier controlled thermostat cell holder (PTC-423S). CD titrations were performed at 20°C. A rectangular quartz cell with 0.1 cm path length was used for the CD studies. Spectra were
recorded at a scanning rate of 20 nm/min and each spectrum was always an average of six scans measured under condition of stirring and was baseline corrected. Nitrogen flushed continuously through the machine at the rate of 5 L min\(^{-1}\). The CD melting profiles were acquired by increasing temperature at the rate of 1ºC/min from 20 to 90ºC. The melting experiments were carried out following the molar ellipticity at 270 nm wavelength. The CD instrument was routinely calibrated with D-camphorsulfonic acid. Data acquisition and analysis were performed on a computer, interfaced to the spectropolarimeter.

**Denaturation experiments**

All the UV-melting curves were generated by measuring absorbance at 260 nm on a JASCO V660 UV-Vis spectrophotometer equipped with a thermoelectrically controlled cell holder and a temperature controller that maintained the temperature inside the sample cell within ±0.1ºC in matched quartz cells of 1cm path length. The melting temperatures determined at half of the maximal hyperchromicity were corresponding within ±1ºC. All other experimental details were provided previously\(^{28}\).

**Isothermal titration calorimetry (ITC)**

ITC experiments were performed using a Microcal VP-ITC titration microcalorimeter (MicroCal, Inc., Northampton, MA, USA) at 25ºC as reported, previously\(^{28}\). Total 28 serial injections of TFO (10 μL, 11 μM) were added at 240 s intervals to DNA duplex (1.5 μM) solution. Control experiments were carried out to calculate the heat of dilution for TFO into buffer. The net enthalpy for TFO-duplex interaction was determined by subtracting the heats of dilution for the buffer from TFO-duplex titration curves. Each experiment was repeated at least four times, and the error values that reflected the standard deviations among the different runs were always less than 10% which indicates the quality of the data.

**Differential scanning calorimetry (DSC)**

DSC measurements were performed with Microcal VP-scanning calorimeter (Micro Cal, Inc., Northampton, MA, USA). In a series of DSC scans, both the cells were loaded with buffer solution, equilibrated at 10ºC for 15 min and scanned from 35 to 100ºC at a scan rate of 60ºC/h. The buffer scans were repeated till reproducible and on cooling, the sample cell was rinsed and loaded with DNA duplex and then with the TFO–DNA duplex and again scanned in the range 35-100ºC. The DSC thermograms of excess heat capacity versus temperature plots were analyzed using the Origin 7.1 software.

**Gel retardation assay (GRA)**

The pyrimidine strands of DNA duplex and respective TFO were 5' end labeled with γ\(^{-32}\)P [ATP] by T4 polynucleotide kinase. Triplex was obtained by mixing 50 nM DNA duplex (with labeled pyrimidine strand) and corresponding TFO in different combination in Sodium Cacodylate buffer at pH 7.4. The mixtures were heated to 95ºC for 10 min followed by slow cooling to 20ºC. The assay was performed as method described earlier\(^{29}\).

**Cell culture and proliferation assay**

Human cervical cancer HeLa cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% (v/v) Fetal Calf Serum, 3.7 g/L sodium bicarbonate and Ciprofloxacin 10 μg/mL at 37ºC. Transfection of TFO to Hela cells was carried out using Lipofectamine™ LTX and PLUS™ reagents following manufacturer’s protocol. Briefly, different concentrations of TFO ranging 0.0-5.0 μM were diluted into 100 μL of Opti-MEM® I reduced serum medium without serum. PLUS™ reagent was mixed gently before use, followed by addition of 0.5 μL PLUS™ Reagent (1:1 ratio) to DNA directly to the diluted DNA. The cells were incubated for 72 h. We elucidated transfection efficiency by checking the GFP expression driven through CMV promoter. Overall, 60-65% transfection efficiency was achieved. Cytotoxicity was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay as described earlier\(^{28}\).

**Flow cytometry**

Cells were plated in 6-well plates, transfected with hmga1-TFO along with control TFO and harvested for 72 h. The cells were collected by centrifugation at 800 rpm for 5 min and fixed with 70% ice-cold ethanol. Cells were resuspended in 100 mL PBS containing 100 mg/mL RNase A and incubated for 15 min at 4ºC. Nuclei were stained by adding 5 μL of propidium iodide solution (50 μg/mL in 0.1% sodium citrate) and analyzed on a BD Biosciences Flowcytometer (BD FACSCanto). Cell cycle distribution and percentage of apoptotic cells were determined using BD FACSDiva software (BD, San Diego, CA, USA)
Protein extraction and Western blot analysis

HeLa cells were grown in different concentrations of \textit{hmga1}-TFO (0.1, 0.3 and 1.0 µM) along with control TFO and total cell protein was extracted using radiounprecipitation assay buffer (150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1% Triton X-100 and 5 mM Tris (pH 8.0) and concentration was measured using the BCA assay kit with bovine serum albumin as a standard. Further, expression levels of HMGA1 were determined by Western blot analysis as described previously\textsuperscript{28}. Equal amount of 25 µg of protein from untreated cells as control and TFO treated cells was loaded and electrophoresed on 15% SDS gel. Protein bands were blotted onto nitrocellulose membrane using a Bio-Rad transfer apparatus. The membrane was blocked with 5% (w/v) non-fat milk powder in TBS containing 0.1% tween-20 (TBS-T) for 30 min and later incubated with HMGAl antibodies. The mouse polyclonal anti-HMGA1 (1:5000) was diluted in 2% BSA in TBS-T and incubated overnight at 4°C, which was followed by an anti-mouse antibody conjugated with horse radish peroxidase. Antibody-reactive bands were detected using the enhanced chemiluminescence Western detection kit (Femto Lucent).

RNA isolation and RT-PCR

Total RNA was isolated from HeLa cells following TFO treatment at 72 h by using Trizol reagent (Invitrogen Laborotories, Calsbad, CA) and 1.5 µg RNA was used to synthesize the first-strand cDNA by SuperscriptsIII RT (Invitrogen) using a standard protocol. PCR amplification was carried out in a 20 µL containing 1 µL of cDNA, 10X PCR buffer, 10 mM each deoxynucleotide triphosphates, 1U of taq DNA polymerase and 10pM of each primer was added. The human \textit{hmga1} primers used were the forward primer 5’-AAC CAG CAC AAG TCC AGG AGG G-3’ and the reverse primer 5’-AAA GCT GTC CAG TCC AGG G-3’. As a control reaction for intact RNA and cDNA, PCR amplification of the house keeping gene \textit{β-actin} was performed on all samples to normalize the sample amount. The forward primer 5’-ATG ACG ATA TCG CTG CGC TGG T-3’ and the reverse primer 5’-AAT ACA GCC CGG GGA GCA TCG T-3’ were used for detecting human \textit{β-actin}. The detailed protocol of the experiment has been described, previously\textsuperscript{28}.

Results

Selection of triplex target sites

The triplex target site (TTS) in \textit{hmga1} promoter was selected using Triplex forming oligonucleotide target sequence search tool (MD Anderson Method). The potent TTS (24 bps) lies in the proximal regulatory region 2 (PRR2) of \textit{hmga1} promoter as shown in Fig. 1. The triplex formed by TFO and corresponding TTS is illustrated in the lower part of figure 1.

Conformational analysis of DNA triplex

Circular dichroism (CD) spectrum of DNA triplex (Tp) which showed a broad positive CD bands at
around 270 nm and a negative CD band at 245 nm is shown in Fig. 2. The CD spectrum of Tp provides a characteristic negative peak at around 210 nm which is characteristic feature of DNA triplex (Fig. 2).

**Biphasic melting transition of DNA triplex**

The first derivative profile of UV melting of Tp is shown in Fig. 3A. The result demonstrates biphasic melting in two sequential well resolved steps, termed as \( T_{m1} \) and \( T_{m2} \), which establishes the presence of two DNA species in the system, first is the low temperature transition (\( T_{m1} \), 53.0±1.0°C) which corresponds to the triplex to duplex transition while the second is higher temperature transition (\( T_{m2} \), 68.0±0.5°C) which corresponds to the denaturation of duplex to single strands. The first derivative of CD melting profile has been depicted in Fig. 3B. The CD melting profile represents two transitions. The first transition of Tp is centred around 52±1.0°C (\( T_{m1} \)) while, the second transition of value 69±0.5°C (\( T_{m2} \)) is accompanied by a large ellipticity change and corresponds to the dissociation of the duplex to single strands.

**Thermodynamic parameters of triplex formation**

Heat change of DNA triplex formation was measured using ITC as shown in Fig. 4. The heat burst of each curve corresponding to a single TFO injection into duplex is shown in Fig. 4 (upper panel). The area under these heat burst curves were determined by integration to yield the associated injection heats. These injection heats were corrected by subtracting the corresponding dilution heats derived from the injection of identical amounts of TFO into buffer alone. The resulting corrected heats plotted as a function of molar ratio is shown in the lower panel of Fig. 4. The data were fitted to the single set of identical sites model that yielded a fairly reasonable fitting of the experimental data. The binding data showed a single strong exothermic binding event and resulted an enthalpy change, \( \Delta H = -91.16±0.3 \) kcal M\(^{-1}\), entropy term change, \( T \Delta S = 81.05±0.1 \) kcalM\(^{-1}\) and binding affinity (Ka) of 5.08 \( \times 10^7 \) M\(^{-1}\). We further investigated the binding stability of TFO to duplex using DSC melting profile (Cp versus T) and the result is presented in Fig. 5. DSC results revealed biphasic melting transition of Tp. The triplex was shown to melt noncooperatively and irreversibly (van’t Hoff enthalpy, \( \Delta H_v = 1.9 \times 10^2±0.7 \) kcalM\(^{-1}\) and the calorimetric enthalpy, \( \Delta H_c = 0.51\times10^2 \) kcalM\(^{-1}\).
±3.2 kcalM⁻¹) with $T_m^1$ of 56.33±0.15°C, while duplex melted cooperatively and reversibly ($\Delta H_v = 1.07 \times 10^2$ ±1.8 kcalM⁻¹ and $\Delta H_c = 1.16 \times 10^2$±2.5 kcal M⁻¹) with $T_m^2$ of 68.67±0.028°C.

We further exploited gel retardation assay (GRA) using radioactive $\gamma$-32P [ATP] to confirm DNA triplex formation. Autoradiogram of GRA of 50 nM duplex alone (lane 1) and mixtures of duplex and TFO in different mole ratios, 2:1 (lane 2), 1:1 (lane 3) and 1:2 (lane 4) is demonstrated in Fig. 6. Gel result shows two bands, one the faster moving (lower band) corresponding to the DNA duplex and the retarded one (upper band) which represents the DNA triplex.

**Effect of TFO cell cycle progression and apoptosis in HeLa cells**

In order to measure growth inhibition in response to TFO, we investigated DNA content and cell cycle distribution in HeLa cells under untreated and TFO-treated conditions using flow cytometry. Taking into account of the resistance towards exogenous and endogenous nucleases, the TFOs were phosphorothioated in their sugar-phosphate backbone. Fig. 7 (A-E) shows the results of the analysis of cells transfected with different concentration of $hmga1$-TFO (0.1, 0.3 and 1.0 µM) along with control TFO (0.3 µM) and grown for 72 h. The population of apoptotic cells in TFO treated sample was 31.8%, while in untreated samples it was 6%. In addition, the fraction of G0/G1 cells was decreased in TFO treated sample (60.7%-52.7%) at 1.0 µM concentration with corresponding decrease in the percentage of cells in S phase (16.8-8.4%).

**TFO mediated reduction of expression of $hmga1$ gene**

In order to determine the effect of $hmga1$-TFO on $hmga1$ expression, Hela cells were transfected with different concentrations (0.1µM, 0.3µM and 1.0µM) of TFO along with control TFO and RT-PCR was performed at 72hr. Fig. 8A shows a concentration dependent reduction of $hmga1$ RNA level with a
maximum reduction observed at a concentration of 1.0µM, while expression of ‘housekeeping gene’ \( \beta\)-actin remained essentially unaltered. RT-PCR result showed downregulation (~48%) of \( \text{hmga1} \) in HeLa cells following \( \text{hmga1}-\text{TFO} \) treatment at IC50 of 0.3 µM at 72 h. The corresponding densitometric analysis is given in Fig. 8B. The control TFO had no effect on \( \text{hmga1} \) transcription and levels of \( \beta\)-actin mRNA were similar in all samples. To confirm that reduction of \( \text{hmga1} \) transcription, would result in lower level of HMGA1 protein, we performed Western blot analysis of cells treated with \( \text{hmga1}-\text{TFO} \) together with control TFO. As shown in Fig. 9A, the HMGA1 level was found to be reduced by ~48% at IC 50 at 72 h in the presence of \( \text{hmga1}-\text{TFO} \). The corresponding densitometric analysis is shown in Fig. 9B. \( \beta\)-actin protein levels were remained unchanged.

**Discussion**

In order to achieve anti-gene strategy of gene regulation, it is important to look for potent target sequence in the gene and respective design of TFO. We have used triplex forming oligonucleotide target sequence search tool to search triplex target sequence (TTS) in \( \text{hmga1} \) promoter27. The TTS of 24 bps was selected from promoter because homopurine sequences are generally found in gene regulatory regions of promoter and frequently, overlie transcription factors binding sites and are reasonably easy targets of TFO.
Thus, purine rich sequences provide the impression that these are associated with control of gene expression and reasonably easy targets of TFO. Moreover, the sequence of the TFO obviously is aimed to form a greater interaction with the duplex generated by the TTS. Anti-parallel triplex, where TFO and TTS are in the opposite direction is more favourable than that of parallel triplex. Therefore, TFO sequence was designed to form anti-parallel triplex allowing guanine recognition with G:C base pairs in the target, thus forming G:G-C triads, and adenine recognition with A:T base pairs forming A:A-T triads. The schematic representation of *hmga1* promoter along with sequences of target sites is shown in Fig. 1.

Circular Dichroic (CD) spectroscopy study was exploited to gain insights into the conformational changes accompanying DNA denaturation and renaturation. CD spectrum of Tp shows characteristic negative peak at around 210 nm and a broad positive peak at around 270 nm (Fig. 2). While, the 270 nm peak represents DNA duplex, the negative band at around 210 nm is signature peak for triplex formation. Triplexes formed by various sequences give different CD spectra as reported by various groups. The spectroscopic data provides structural insights of molecular interaction. It provides only a partial description of triplex formation. Melting data demonstrated biphasic transition in two sequential well resolved steps, termed as *Tm*1 and *Tm*2 which clearly indicates the formation of the ternary complex. It further establishes that there indeed exist two different species with different physical behaviour. Triplex is formed by Hoogsteen bonding and the interaction of triplex is weak due to higher phosphate electrostatic. This is because of the closer proximity of the phosphates into the parallel chains. Hoogsteen bonds having lower energy in comparison to the Watson-Crick base pairing, *Tm* values of Tp was found to be lower than that of corresponding duplex (Fig. 3). DNA melting was monitored in terms of changes in ellipticity which support the UV melting analysis. The complexity of the nearest-neighbour pattern makes it difficult to distinguish the contribution of hydrogen-bond interactions of bases in triplex and base stacking. These facts seem to play important role in total interaction of TFO and duplex. The factors like stacking interactions, location of the third major groove of DNA leads to changes in the thermodynamic properties as well. Therefore, it is essential to elucidate the thermodynamic data for the complete understanding of the nature of triplex formation. Therefore, we exploited two calorimetric techniques i.e. (i) isothermal titration calorimetry (ITC) and (ii) differential scanning calorimetry (DSC).

ITC and DSC provide ample understanding of thermodynamic mechanism that rules the conformational equilibrium of macromolecules. Ming-Tsai Weya et al., reported similar order of thermodynamic parameters of triplex formation between a segment of cell cycle protein *cdc25* in *Pneumocystis jirovecii*, its complementary and a 17nt TFO. However, it appears that characteristic differences in the binding affinity are thought to be arising from the nucleotide content and length of TFO.

Fig. 9 — (A) Western blot analysis of HMGA1 protein in presence of TFO in HeLa cells at different concentrations. Lane 1 represents control sample, while lane 2 corresponds to non-specific TFO (0.3 µM) treated sample. Lanes 2, 3 and 4 represent specific TFO at 0.3 and 1 µM, respectively at 72 h. Blots were probed with the specific mouse polyclonal anti HMGA1 protein. Antibody reactivity was detected using Femto LUCENT reagent after 1:5000 dilution of the secondary HRP-conjugated antibody. β-ACTIN was loaded as internal control. (B) Densitometric analyses of HMGA1 are shown in the bar diagram. The bars correspond to mean ±SD values.
and the variation in flanking sequence of targeted region in gene. Melting temperature obtained from DSC analysis corroborated UV and CD melting studies of DNA triplex. DSC data further demonstrates that ratio of calorimetric to van’t Hoff enthalpy for the DNA duplex melting is unity indicating two state transitions, i.e. the transition occurs in all or none fashion, without any intermediate state. The energetic data further interpret that the binding of TFO to DNA duplex was found to be exothermic and favored by both negative enthalpy and positive entropy changes. Moreover, biochemical method using GRA confirmed the DNA triplex formation. GRA is based on the observation that stable DNA-ligand complexes migrate through polyacrylamide gels more slowly than free DNA fragments and helps to visualize the high molecular weight triplex. The autoradiogram of GRA of duplex alone and complex of duplex and TFO in different ratios revealed two bands, one the faster moving (Lower Band) corresponding to the duplexes and the retarded (upper band) one representing triplexes (Fig. 6). As, the TFO ratio in the mixture is increased, the intensity of the low mobility band is found to be increased suggesting more of triplex formation. The result strongly affirms the finding reported by Mergny JL et al., who demonstrated DNA triplex formation using ethidium bromide.

We further studied the in-vitro effect of TFO on HMGA1 expression, keeping in view that it will assess the correlation between the affinity of binding of TFO to hmga1 target site and its direct inhibitory effect on the transcription of hmga1. Expression study provides the impression that antiproliferative and antigene effect of TFO resides in the binding specificity. Phosphorothioated hmga1-TFO was used taking into account of exogenous and endogenous nucleases. Control TFO, which was non-specific and non-selective to the target sequence, did not show any anti-proliferative effect on the cells. Expression of HMGA1 was significantly reduced in presence of hmga1-TFO as compared to that of control TFO. Subsequently, the apoptotic cell population in response to hmga1-TFO was higher even at lower concentration of IC50 value (0.3µM). The control TFO had no effect on hmga1 transcription and levels of β-actin mRNA were similar in all samples, suggesting that the effect of TFO was specific and selective for hmga1 gene (Fig. 8). β-ACTIN levels were remained unchanged, thereby demonstrating equal protein loading and absence of nonspecific inhibition of protein synthesis in transfected cells (Fig. 9). The finding is supported by TFO mediated inhibition of other genes i.e. c-myc, survivin, rhodopsin. The appearance of sub-G1 population indicated that incubation of cells with hmga1-TFO increased the rate of apoptotic cell death and probably accounted for the loss of cells from G1 compartment. Carbone et al., reported the similar sub-G1 population in human prostate cancer cells in presence of TFO. TFO mediated inhibition of gene expression is based on interplay between transcriptional activators, corepressors and general transcription factors. Transcription process gets initiated with the binding of transcriptional activators resulting in recruitment of transcription factors, like TATA-binding protein (TBP) and TBP-associated factors (TAFs), to the promoter. The general transcription factors along with RNA polymerase II (RNAPII) form the pre-initiation complex (PIC) leading to formation of the active open complex. For genes which lack TATA box like hmga1, binding of transcriptional activators like Sp1 near the transcription start site, and its interaction with the TFIID complex are essential for the assembly of the PIC and transcriptional activation process. Depending on the position of the triplex target site relative to the TSS, TFO could prevent, either directly or indirectly, the binding of an activator, RNAPII or other components of the transcriptional machinery and interfere with the assembly of the PIC.

**Conclusion**

The present study demonstrates that hmga1-TFO forms stable triplex with the sequence of the human hmga1 promoter as revealed by biphasic melting transition of triplex, conformational analysis and mobility shift assay. Thermodynamics data reveal that the interaction between TFO and the hmga1 gene sequence to be exothermic in nature and is favoured by both negative enthalpy and positive entropy changes. The study further underlines the in-vitro triplex formation in correlation with its effect on HMGA1 expression. The hmga1-TFO significantly downregulates the expression of HMGA1, inhibits proliferation and induces apoptosis in cervical cancer cells. These findings establish the role of TFO as a potential anticancer agent and a promising tool to modulate gene expression and inhibit survival of cancer cells.
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